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14. ABSTRACT This is a progress report (year 2) for the development of a novel therapeutic drug (salubrinal) for skeletal diseases, focusing on potential treatment of osteoporosis and bone fracture. The study in the second year using animal models and *in vitro* cell cultures strongly supported salubrinal's action on prevention of bone loss. In animal experiments, we employed three procedures to induce bone loss. They were ovariectomy, hindlimb suspension, and administration of glucocorticoid (prednisolone). In all three models, salubrinal was able to suppress reduction of bone mineral density. In those experiments, salubrinal was administered via subcutaneous injection as well as oral gavage. In *in vitro* experiments, salubrinal reduced the development of bone-resorbing osteoclasts and promoted the development of bone-forming osteoblasts. A provisional patent was submitted for salubrinal's administration for treatment of bone diseases, and the peer-reviewed articles as well as conference abstract were published. In the third year, we will start evaluating the effects of salubrinal on the healing of bone fracture as well as the regulatory mechanism of salubrinal's action.						
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A. Introduction

This is a second-year progress report of the project (W81XWH-11-1-0716; Development of a novel synthetic drug for osteoporosis and fracture healing). The project aims at developing a novel synthetic drug for osteoporosis and healing of bone fracture, and a particular focus is usage of salubrinal, a synthetic chemical agent ($C_{21}H_{17}Cl_3N_4OS$, 480 Da), as a novel drug candidate.

The statement of work (SOW) was revised and approved during the second year. The animal procedure was also revised and approved in the second year. Based on the revised SOW, the second year was focused on the following subtasks in Tasks 1, 2, 3, and 5:

- **Task 1** – Develop formulations for four routes of administration (oral, subcutaneous, intravenous, and local immobilization in implantable matrix), and determine pharmacodynamics
    - Subtask 1d. Determine circulating levels of the drug and evaluate pharmacodynamics
    - Subtask 1e. Evaluate cellular responses using ovariectomized mice and hindlimb suspended mice

- **Task 2** – Evaluate the efficacy of salubrinal on bone formation in young and ovariectomized mice compared to the anabolic agent PTH.
    - Subtask 2b. Evaluation of subcutaneous administration using ovariectomized mice
    - Subtask 2c. Evaluation of oral gavage administration using ovariectomized mice

- **Task 3** – Evaluate the efficacy of salubrinal in preventing bone loss induced by glucocorticoid excess in mice compared to administration of alendronate.
    - Subtask 3a. Comparison of salubrinal and alendronate

- **Task 5** – Develop a commercialization plan.
    - Subtask 5b. Partnership selection

This progress report documents the results obtained in the second year on the above subtasks. Note that Task 4 is planned to be conducted in the third and fourth years.

B. Body

Task 1: Develop formulations for four routes of administration (oral, subcutaneous, intravenous, and local immobilization in implantable matrix), and determine pharmacodynamics

Subtask 1d. Determine circulating levels of the drug and evaluate pharmacodynamics

We evaluated the salubrinal concentration in the serum in response to oral gavage (Fig. 1). The peak concentration appeared in 30 min, and in 2 h the concentration was close to the background noise level (N = 2 - 3 for each of eight time points).

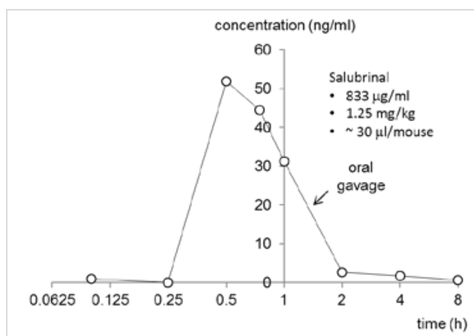

Fig. 1. Pharmacokinetics data for salubrinal administered via. oral gavage.

Subtask 1e. Evaluate cellular responses using ovariectomized mice and hindlimb suspended mice

Ovariectomized mice: Using bone marrow derived cells harvested from the ovariectomized (OVX) mice, we examined the effects of salubrinal on development of bone-resorbing osteoclasts and bone-forming osteoblasts. Three groups are the sham OVX control, OVX treated with vehicle, and OVX treated with salubrinal (N = 10 per group). Four weeks after ovariectomy, mice received daily salubrinal administration (s.c., 1 mg/kg body weight) for 4 weeks and bone marrow cells were harvested from a pair of femora.

Regarding the development of osteoclasts, we conducted 4 assays (N = 3 for each assay):

- CFU-M (colony-forming unit – macrophage): evaluation of proliferation of pre-osteoclasts
- Area covered by osteoclasts: evaluation of maturation of multi-nucleated osteoblasts
- Migration: evaluation of migration of osteoclasts
- Adhesion: evaluation of adhesion of osteoclasts

In all assays, the results showed that OVX treatment elevated development of osteoclasts and the OVX-driven elevation was suppressed by administration of salubrinal (Fig. 2).

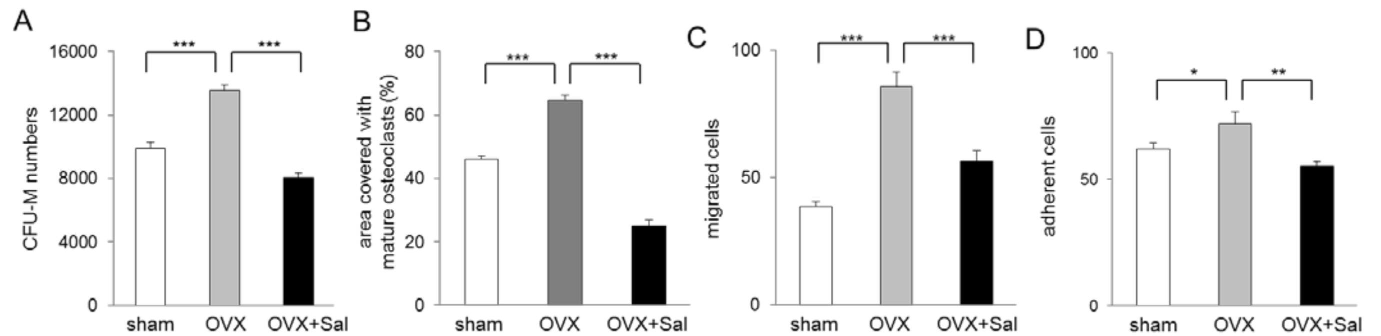

Fig. 2. Effects of salubrinal on development of osteoclasts using bone marrow cells harvested from the OVX mice. (A) Salubrinal-driven suppression of CFU-M. (B) Salubrinal-driven suppression of development of multi-nucleated osteoclasts. (C) Salubrinal-driven suppression of migration of osteoclasts. (D) Salubrinal-driven suppression of adhesion of osteoclasts. Note that the single, double, and triple asterisks indicate $p < 0.05$, 0.01, and 0.001, respectively.

Regarding the development of osteoblasts, we conducted 2 assays (N = 3 for each assay):

- CFU-F (colony-forming unit – fibroblast): evaluation of abundance of mesenchymal stem cells using fibroblasts as a cellular marker
- CFU-OBL (colony-forming unit – osteoblast): evaluation of development of osteoblasts

In both assays, OVX treatment did not significantly alter the numbers in CFU-F and CFU-OBL. However, administration of salubrinal increased CFU-F and CFU-OBL (Fig. 3).

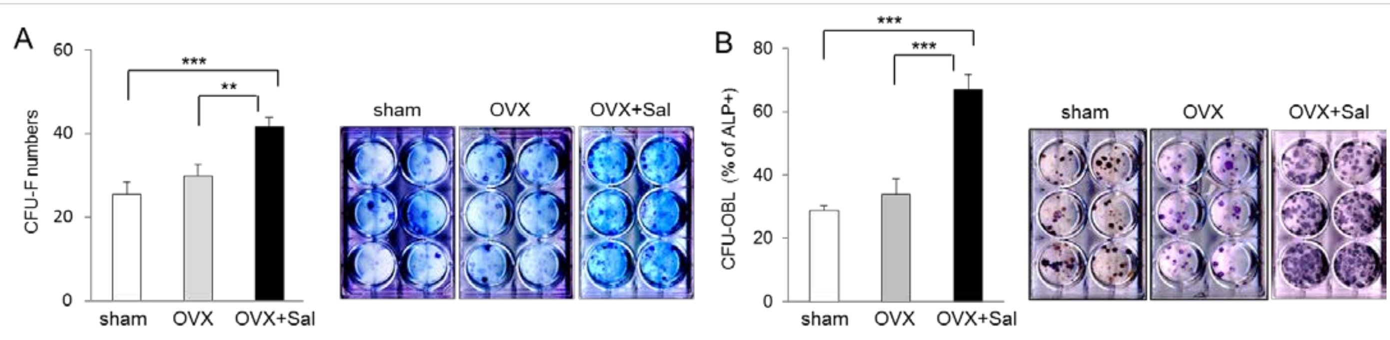

Fig. 3. Effects of salubrinal on development of fibroblasts and osteoblasts. (A) Salubrinal-driven elevation of CFU-F (fibroblasts). (B) Salubrinal-driven elevation of CFU-OBL (osteoblasts).

The result in Figs. 2 and 3 provide evidence that administration of salubrinal to OVX mice inhibits bone-resorbing osteoclasts and promotes bone-forming osteoblasts.

Hindlimb suspended mice: OVX treatment is one procedure to induce bone loss in a global fashion, and hindlimb suspension is a popular procedure to reduce bone mass in the hindlimb. In hindlimb suspension, mice received unloading to their hindlimb for 2 weeks and salubrinal was administered (s.c.) daily concurrently (N = 10 for vehicle and N = 10 for salubrinal). Bone marrow cells were harvested from the femora after 2 week hindlimb suspension.

First, we examined the effects of salubrinal on BMD (bone mineral density) and BMC (bone mineral content) of the lumbar spine, femur, and tibia. The results showed that in all three regions administration of salubrinal significantly increased BMD and BMC (Fig. 4).

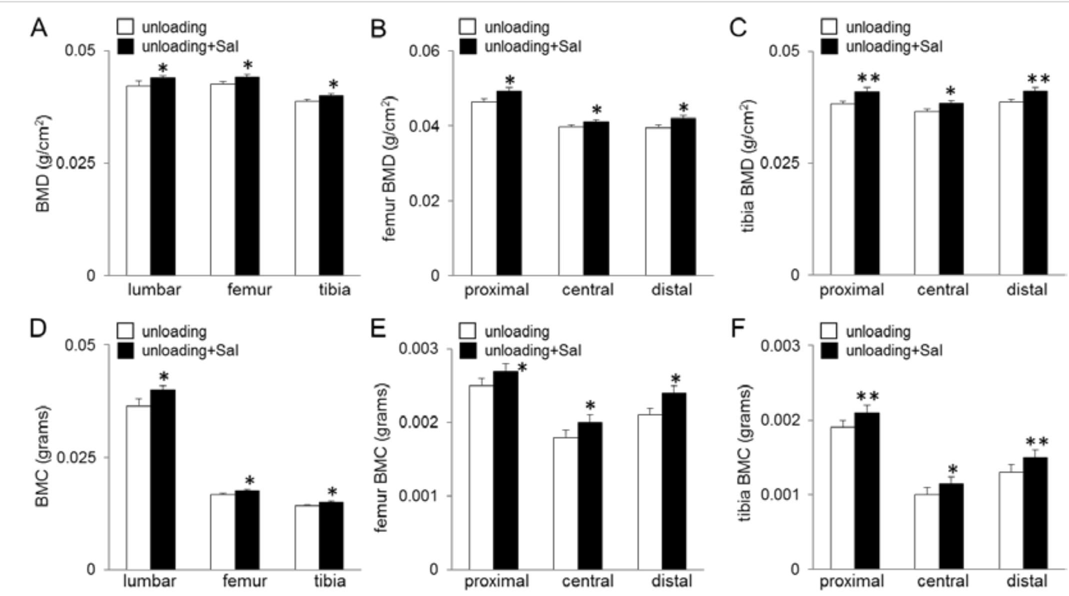

Fig. 4. Elevation of BMD (bone mineral density) and BMC (bone mineral content) by salubrinal in the unloaded mice. (A) BMD in the lumbar spine, femur and tibia. (B-C) BMD in the femur and tibia, respectively. (D) BMC in the lumbar spine, femur and tibia. (E-F) BMC in the femur and tibia, respectively.

Regarding the development of osteoclasts and osteoblasts, we conducted an osteoclast maturation assay (Fig. 5A) and CFU-OBL (Fig. 5B). Consistent with the results for BMD and BMC, hindlimb suspension elevated the area covered by osteoclasts and reduced the number in CFU-OBL. Administration of salubrinal, on the contrary, reduced the development of multi-nucleated osteoclasts and increased the number of CFU-OBL.

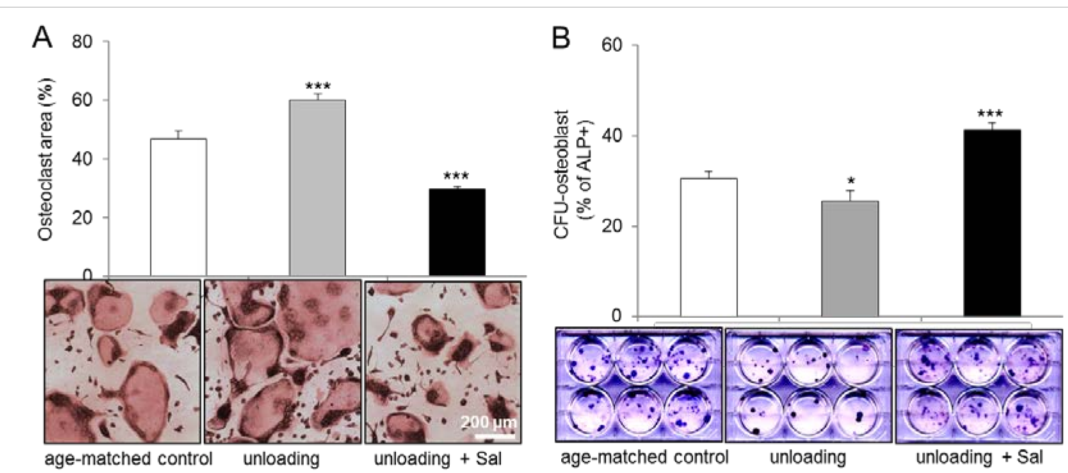

Fig. 5. Suppression of unloading-induced changes to osteoclasts and osteoblasts by salubrinal. (B) Suppression of unloading-driven increase in osteoclast development by salubrinal. (C) Suppression of unloading-driven decrease in CFU-osteoblast by salubrinal.

The results with hindlimb suspended mice confirmed that administration of salubrinal suppressed the development of osteoclasts and promoted the development of osteoblasts.

Task 2: Evaluate the efficacy of salubrinal on bone formation in young and ovariectomized mice compared to the anabolic agent PTH (parathyroid hormone)

Subtasks 2b. Evaluation of s.c. administration using ovariectomized mice
Using the OVX mice, we determined body weight, uterus weight, BMD and BMC and evaluated the effects of salubrinal that was administered daily via s.c. (1 mg/kg). OVX treatment elevated body weight, and reduced uterus weight, and BMD and BMC of the lumbar spine, femur and tibia (Fig. 6). Administration of salubrinal suppressed OVX-induced changes in body weight, uterus weight, and BMD/BMC (Fig. 7). In particular, BMD and BMC of the lumbar spine, femur, and tibia were significantly increased by salubrinal ($p < 0.001$).

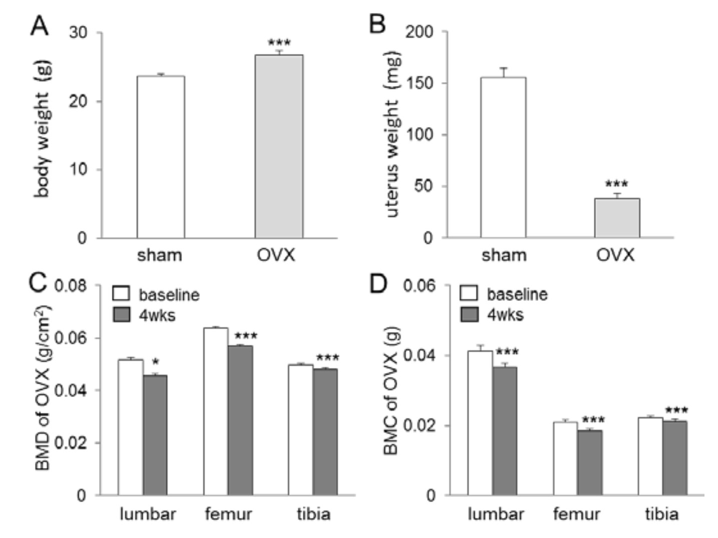

Fig. 6. Effects of OVX treatment. (A) Body weight. (B) Uterus weight. (C) BMD in the lumbar spine, femur and tibia. (D) BMC in the lumbar spine, femur and tibia.

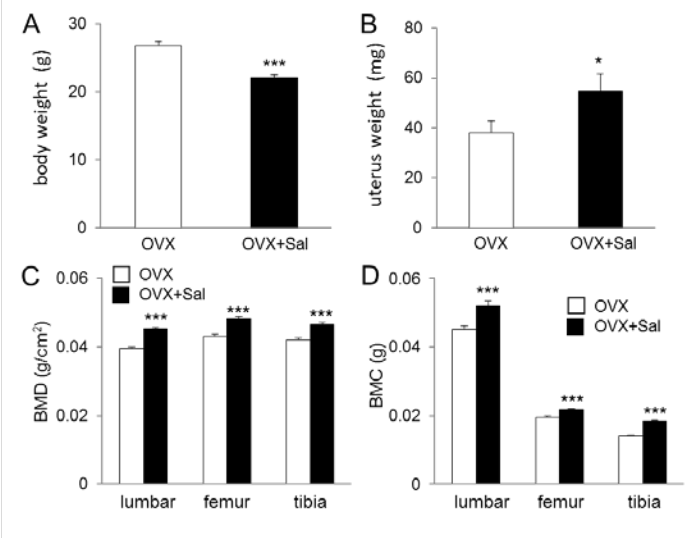

Fig. 7. Effects of Salubrinal to OVX mice. (A) Body weight. (B) Uterus weight. (C) BMD in the lumbar spine, femur and tibia. (D) BMC in the lumbar spine, femur and tibia.

Subtask 2c. Evaluation of oral gavage administration using ovariectomized mice
We also examined the effects of salubrinal when it was administered orally, and the results were compared with the effects of PTH administration. In this experiment, a group of mice received OVX surgery and the other group sham OVX surgery (the operation without removing ovaries) (N = 10 per group). Mice in the salubrinal group received daily administration of salubrinal via oral gavage, which mice in the PTH group received PTH (0.1 mg/kg) via subcutaneous injection (positive control). The BMD and BMC of the femur, tibia, and lumbar spine were measured by pDEXA. Administration of salubrinal enhanced BMD/BMC in both sham OVX and OVX mice, and OVX mice were more sensitive to salubrinal than sham OVX (Fig. 8A & B). PTH increased BMD/BMC in both sham OVX and OVX. Unlike the effects of salubrinal, sham OVX mice presented a higher sensitivity to PTH than OVX mice (Fig. 8C & D).

Using micro-CT images of the femora obtained with SkyScan, we evaluated bone volume and trabecular parameters such as BV/TV (ratio of the mineralized bone volume to the total volume), Tb.N (trabecular number), Tb.Th (trabecular thickness), and Th.S (trabecular separation). Compared to the OVX vehicle control, subcutaneous injection of salubrinal increased the parameters BV/TV, Tb.N, and Tb.Th (all $p < 0.05$), and decreased Tb.S ($p < 0.05$) (Fig. 9). The result indicates that oral administration of salubrinal prevents bone loss not only in cortical bone but also in trabecular bone.

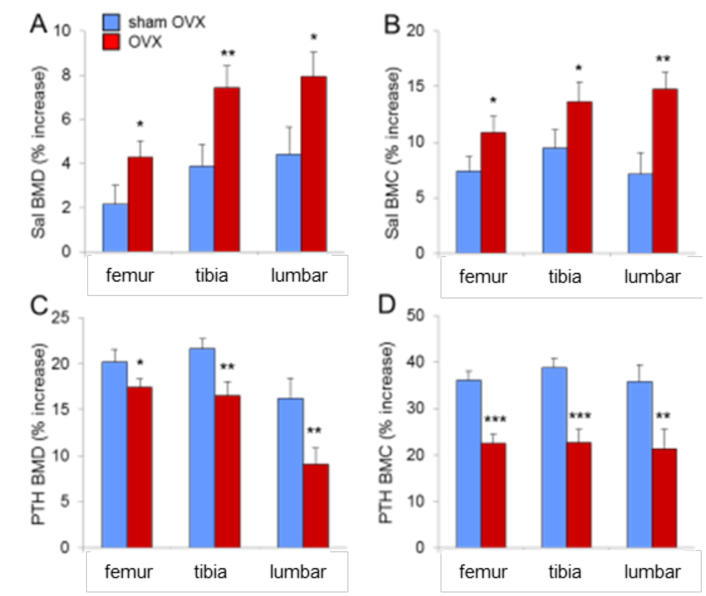

Fig. 8. Effects of salubrinal (oral administration) and PTH on BMD and BMC. (A-B) Increase in BMD and BMC by salubrinal. (C-D) Increase in BMD and BMC by PTH.

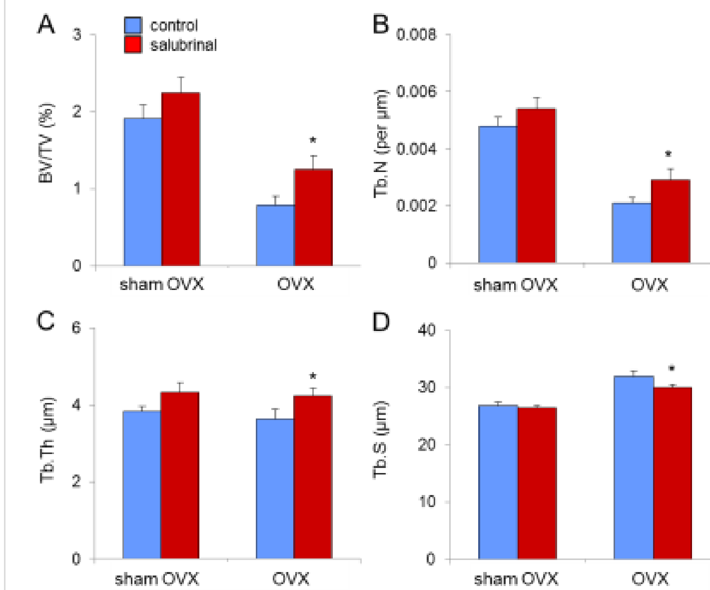

Fig. 9. Effects of salubrinal (oral administration) on bone parameters to OVX mice. (A) Increase in BV/TV. (B) Increase in Tb.N. (C) Increase in TB.Th. (D) Reduction in Tb.S.

Task 3: Evaluate the efficacy of salubrinal in preventing bone loss induced by glucocorticoid excess in mice compared to administration of alendronate

Subtask 3a. Comparison of salubrinal and alendronate

We evaluated the effects of salubrinal using mice that received systemic administration of glucocorticoid (GC; prednisolone). Prednisolone (two dosages: GC1 – 1.4 mg/kg, and GC2 – 2.1 mg/kg) and placebo pellets (Innovative Research of America) were subcutaneously implanted in the back neck region (N=7–10 per group). Daily salubrinal administration (s.c., 1 mg/kg) was conducted for 4 weeks, and alendronate (5.25 mg/kg/week) was treated as positive control. The changes in BMD (total, spine, and femur) are shown (Fig. 10).

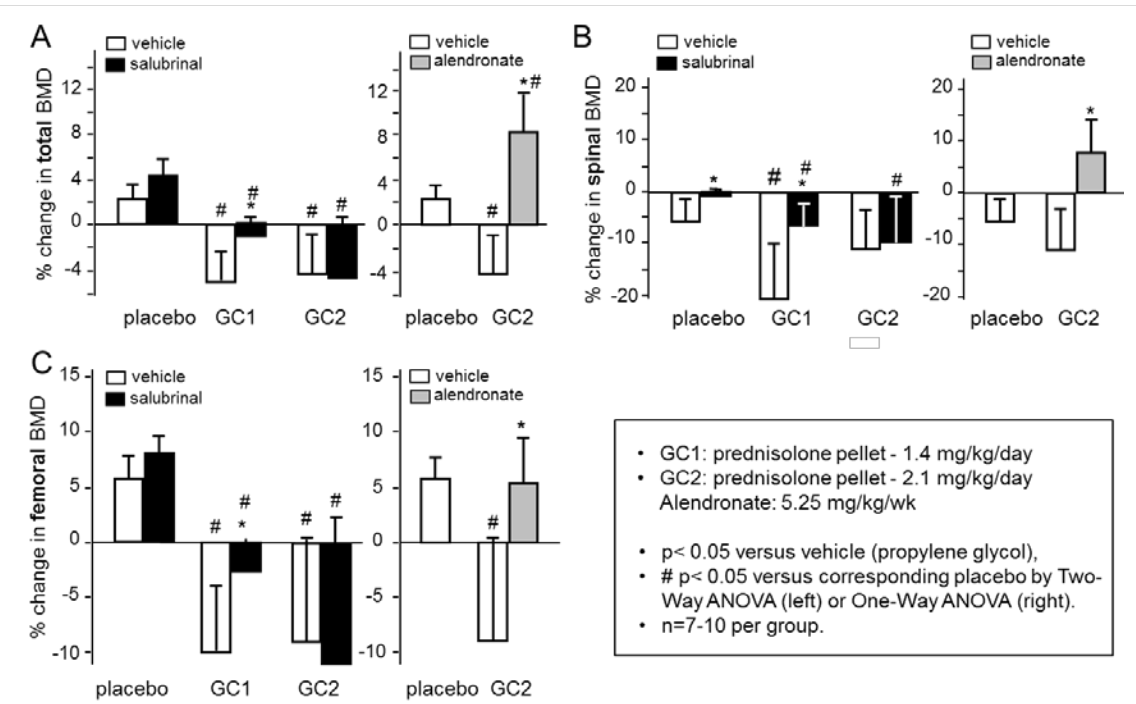

Fig. 10. Effects of salubrinal to glucocorticoid (GC; prednisolone) treated mice. Note that alendronate is used as a positive control. (A) Percent change in BMD o the total body. (B) Percent change in the spinal BMD. (C) Percent change in the femoral BMD.

Treatment with GC1 and GC2 significantly reduced BMD (total body, femur, and spine) except for the spine with GC2, but no clear dose response was obtained. For GC1, salubrinal suppressed GC1-driven loss of BMD in the total body, spine, and femur. For GC2, salubrinal's effect was not statistically significant.

To evaluate the effects of salubrinal on bone resorption and formation, we determined the level of three serum markers (CTX: C-telopeptide fragments of type I collagen, OCN: osteocalcin, and ALP: alkaline phosphatase) (Fig. 11). CTX is a marker for bone resorption, while OCN and ALP for bone formation. The blood was collected from the facial vein of mice after 3-h fasting. The concentrations of OCN and CTX were measured using an enzyme-linked immunosorbent assays (Biomedical Technologies, and Immunodiagnostic systems and Immunodiagnostic Systems, respectively), and ALP with the AMP Buffer (Randox # AP 3802) method using a Randox Daytona analyzer (Randox Laboratories). Because of variations among mice (n = 7 to 10), the result did not show any clear trend either for salubrinal or alendronate except for the result of CTX with GC2 and alendronate.

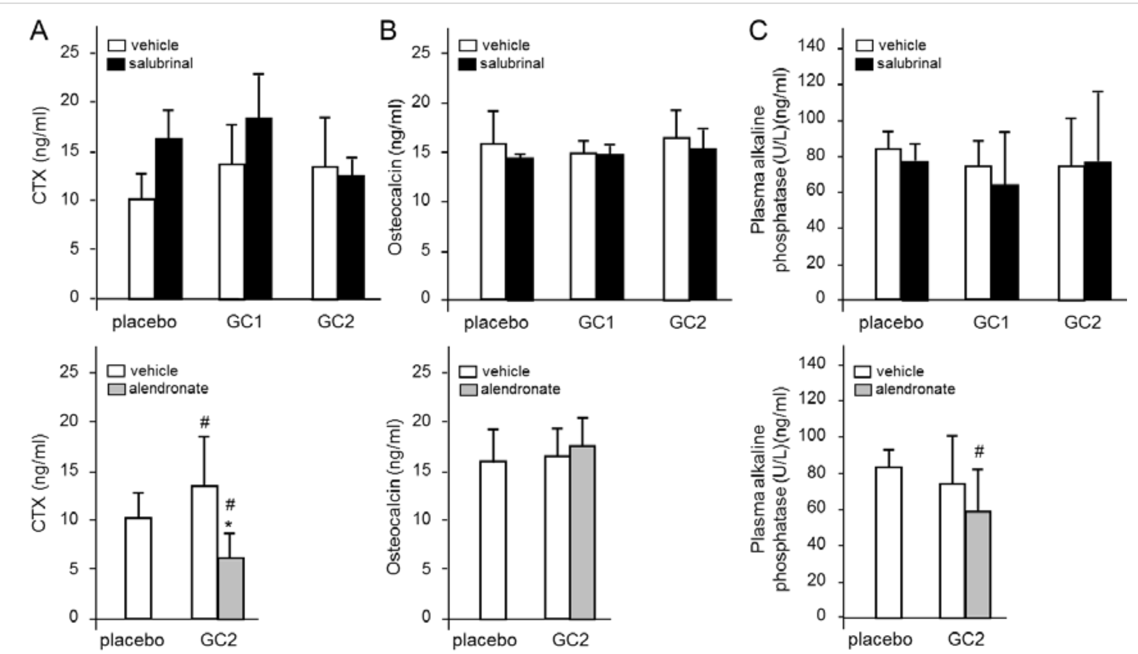

Fig. 11. Effects of salubrinal and alendronate on the serum markers. Note that alendronate is used as a positive control. (A) CTX in the serum. (B) Osteocalcin in the serum. (C) Alkaline phosphatase in the serum.

Using an ElectroForce 3100 actuator, we evaluated stiffness of the L6 lumbar vertebra. Compressive loading was imposed using a ramp function applied at 0.5 N/s to a peak compressive force of 6 N. The result showed a trend that treatment of GC1 and GC2 reduced stiffness and administration of salubrinal and alendronate increased it, although no statistical significance was obtained with N = 7 to 10 (Fig. 12).

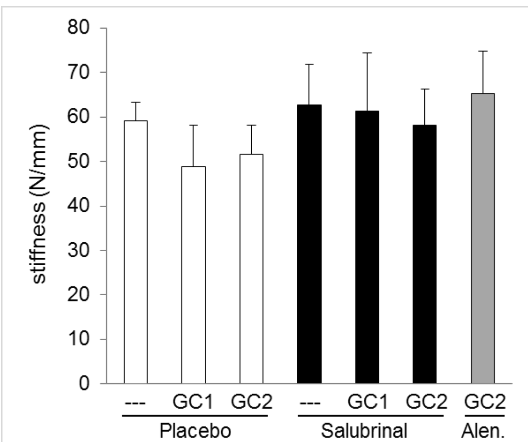

Fig. 12. Effects of salubrinal and alendronate on stiffness of L6 vertebral body. Note that GC1: 1.4 mg/kg/day prednisolone; GC2: 2.1 mg/kg/day prednisolone; and Alen: 5.25 mg/kg/week alendronate.

In summary, the results for mice treated with glucocorticoid showed that salubrinal is capable of suppressing prednisolone-induced reduction of BMD in the whole body, and specifically the lumbar spine and femur. Although data do not show statistical significance, the observed trend is that stiffness of the spine (L6) is reduced by prednisolone and restored by administration of salubrinal.

Task 4: Evaluate the efficacy of salubrinal on bone fracture healing applied locally in matrix sheets compared with BMP2

The task 4 is scheduled to be conducted in Years 3 and 4.

Task 5: Develop a commercialization plan

Subtask 5b. Partnership selection

We contacted Eli Lilly Company and requested any suggestion to establish a potential partnership. One inquiry was whether salubrinal belongs to a selective estrogen receptor modifier (SERM). In order to test any linkage to estrogen receptor, a separate study was conducted using an internal grant available at Indiana University. This grant (Treatment of Bone Metastasis, PI: Yokota) is directed to test efficacy of salubrinal using estrogen receptor negative cells (4T1 mouse mammary tumor cells). 4T1 mouse mammary tumor cells do not express estrogen receptor, and they do not respond to estrogen. If salubrinal is effective to 4T1 cells, salubrinal is less likely to be a standard SERM. The results indicate that salubrinal is not a SERM. We are exchanging ideas about marketability of salubrinal.

C. Key Research Accomplishments

In the second year of this project, the key research accomplishments include the followings:

- Administration of salubrinal via oral gavage suppressed loss of cortical and trabecular bone, which was caused by ovariectomy surgery.
- Subcutaneous administration of salubrinal to unloaded mice by hindlimb suspension suppressed unloading-induced bone loss.
- Administration of salubrinal to glucocorticoid (GC) -treated mice partially suppressed GC-induced reduction in bone mineral density.
- Bone marrow derived cells, which were harvested from the salubrinal-treated mice, exhibited the attenuated development of bone-resorbing osteoclasts.
- Development of osteoblasts was promoted in bone marrow derived cells, which were harvested from the salubrinal-treated mice.

D. Reportable Outcomes

The study in the second year generated the following reportable outcomes.

- A provisional patent was submitted regarding application of salubrinal to treatment of skeletal diseases (compositions and methods for treating metabolic disorders; US Provisional patent application No. 61/717,312, filed on October 23, 2012).
- One peer-reviewed research article was published, while the other is in press (Refs. 1, 2).
- Three conference abstracts were published (Refs. 3 - 5).
- Two conference abstracts were submitted (Refs. 6, 7).
- An internal campus grant (Treatment of Bone Metastasis, $27,250, 07/01/13-06/30/14) was received for examining whether salubrinal is a SERM and whether salubrinal is effective in treatment of bone metastasis.

E. Conclusion

The study in the second year strongly supported salubrinal's action on the prevention of bone loss. In the animal experiments, salubrinal suppressed he reduction in bone mineral density that was caused by ovariectomy, hindlimb suspension, or administration of glucocorticoid (prednisolone). Administration of salubrinal via subcutaneous injection as well as oral gavage was both effective in the prevention of bone loss. In *in vitro* experiments, salubrinal reduced the development of bone-resorbing osteoclasts and promoted the

development of bone-forming osteoblasts. In the third year, we will start evaluating the effects of salubrinal on bone fracture as well as the regulatory mechanism of salubrinal's action.

F. References

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2. Hamamura, K., Tanjung, N., Yokota, H. (2013). Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha. *J. Bone Miner. Metab*. (in press).
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6. Hamamura, K., Tanjung, N., Hamamura, M., Yokota, H. Inhibition of de-phosphorylation of eIF2 alpha suppresses osteoclastogenesis through downregulation of NFATc1. *Abstract, 2014 annual meeting of Orthopedic Research Society* (submitted).
7. Zhang, P., Chen, A., Abedinpoor, A., Yokota, H. Oral administration of salubrinal stimulates bone remodeling in ovariectomized mice. *Abstract, 2014 annual meeting of Orthopedic Research Society* (submitted).

G. Appendix

- Provisional patent application - compositions and methods for treating metabolic disorders
- Reference 1
- Reference 2
- Reference 3

29920-223136

COMPOSITIONS AND METHODS FOR TREATING METABOLIC DISORDERS

GOVERNMENT RIGHTS

This invention was made with government support under W81XWH-11-1-0716 awarded by the Department of Defense. The government has certain rights in the invention.

TECHNICAL FIELD

The invention described herein pertains to the compounds, compositions, and methods for treating metabolic disorders. In particular, the invention described herein pertains to compounds, compositions, and methods for treating obesity, and post menopausal diseases.

BACKGROUND AND SUMMARY OF THE INVENTION

Metabolic disorders elicit in a variety of forms, including obesity-related and bone-related diseases. In addition, metabolic disorders are often more prevalent and/or more severe in older adults, especially in post-menopausal females. Obesity-related diseases illustratively include excess fat storage, excess fat load, and excess fat production, such as through the recruitment of adipoytes. Bone-related disorders illustratively include osteoporosis and osteopenia. It is well established that bone remodeling is conducted by a coordinated action of osteoclasts (bone resorption) and osteoblasts (bone formation). Osteoporosis and osteopenia reportedly reflect an imbalance of the activities of osteoclasts and osteoblasts.

Osteoporosis and osteopenia are major skeletal diseases that put patients, especially the elderly population, at an increased risk for bone fractures (Syed 2010). Osteoporosis and osteopenia linked bone fracture in the hip joint, for instance, is costly to treat and significantly affects quality of life (Budhia et al. Pharmacoeconomics 2012). Postmenopausal women in particular are prone to osteoporosis and osteopenia (Lai 2011). It is been reported that menopause results in decreased production of estrogen followed by bone resorption (Zaidi 2007). Decreased production of estrogen causes a period of rapid bone loss, which is central to the onset of postmenopausal osteoporosis and osteopenia (Riggs 2002).

Several recently-discovered therapies for osteoporosis include Rank-L inhibitors, PTH fragments, bisphosphonate, and cathepsin K inhibitors. Each of those therapies prevents osteoporotic bone loss either by enhancing bone formation or inhibiting bone resorption, but not via both mechanisms. Mechanical loading has also been used as a therapy for osteoporosis. However, mechanical loading may not induce global effects on bone remodeling. Additional compounds, compositions, and methods are needed for treating post menopausal disease, such as oseoporosis and osteopenia. In addition, compounds, compositions, and methods are needed for treating obesity, including post-menopausal obesity and related disorders.

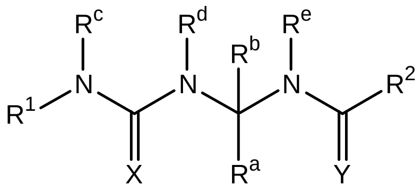
It has been discovered that the compounds described herein are capable of

regulating bone remodeling. Without being bound by theory. it is believed herein that the compounds regulate bone remodeling through the promotion of osteoblast differentiation, such as by increasing the differentiation of progenitor cells, such as bone marrow derived cells, to osteoblasts. In addition, though without being bound by theory. it is believed herein that the compounds regulate bone remodeling through a reduction of osteoclast formation, migration and adhesion, such as by decreasing the differentiation of progenitor cells, such as bone marrow derived cells, to osteoclasts. Compounds described herein are useful for treating postmenopausal diseases, including osteoporosis, uterine weight loss, and post menopausal obesity.

It has also been discovered that the compounds described herein are capable of suppressing increases in body weight and fat storage. Without being bound by theory. it is believed herein that the compounds may regulate weight, fat storage, or fat metabolism, or a combination thereof by decreasing the differentiation of progenitor cells, such as bone marrow derived cells, to adipocytes, and/or by suppressing adipocyte division.

It has also been discovered that the compounds described herein are useful in treating dysuria, such as in controlling urinary urgency and/or urinary frequency. Without being bound by theory, it is believed herein that the loss in uterine weight results in dysuria.

In one illustrative embodiment of the invention, compounds of the following formula are described herein:



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen

in vivo .

In addition, various genera and subgenera of each of X, Y, R^1, R^2, R^a, Rb, R^c, R^d, and R^e are described herein. It is to be understood that all possible combinations of the various genera and subgenera of each of X, Y, R^1, R^2, R^a, Rb, R^c, R^d, and R^e are described herein represent additional illustrative embodiments of compounds of the invention described herein. It is to be further understood that each of those additional illustrative embodiments of compounds may be used in any of the compositions, methods, and/or uses described herein.

In another embodiment, pharmaceutical compositions containing one or more of the compounds are also described herein. In one aspect, the compositions include a therapeutically effective amount of the one or more compounds for treating a patient with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders. It is to be understood that the compositions may include other component and/or ingredients, including, but not limited to, other therapeutically active compounds, and/or one or more carriers, diluents, excipients, and the like. In another embodiment, methods for using the compounds and pharmaceutical compositions for treating patients with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders are also described herein. In one aspect, the methods include the step of administering one or more of the compounds and/or compositions described herein to a patient with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders. In another aspect, the methods include administering a therapeutically effective amount of the one or more compounds and/or compositions described herein for treating patients with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders. In another embodiment, uses of the compounds and compositions in the manufacture of a medicament for treating patients with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders are also described herein. In one aspect, the medicaments include a therapeutically effective amount of the one or more compounds and/or compositions for treating a patient with a metabolic disorder, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders.

It is appreciated herein that the compounds described herein may be used alone or in combination with other compounds useful for treating metabolic disorders, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders, including those compounds that may be therapeutically effective by the same or different modes of action. In addition, it is appreciated herein that the compounds described herein may be used in combination with other compounds that are administered to treat other symptoms of metabolic

disorders, such as but not limited to obesity, bone-related disorders, and post-menopausal disorders, such as compounds administered to treat pain, block dietary fat absorption, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Effects of ovariectomy and salubrinal on body weight. Note that the triple asterisk denotes $p < 0.001$. (A) Gain in body weight by ovariectomy after 8 weeks. The left and right images correspond to the sham OVX and OVX mice, respectively. (B) Reduction in body weight by subcutaneous administration of salubrinal (4 weeks in the second half of the 8-week ovariectomized period) to the OVX mice. The left and right images show the control OVX mouse and the salubrinal treated OVX mouse, respectively.

FIG. 2. Effects of ovariectomy and salubrinal on uterus weight. Note that the triple asterisk denotes ***$p < 0.001$. (A) Reduction in uterus weight in the OVX mice. The images illustrate the sham OVX control mice (left) and OVX mice (right). (B) Increase in uterus weight by subcutaneous administration of salubrinal. The images show the OVX mice (left) and salubrinal-treated OVX mice (right).

FIG. 3. Effects of ovariectomy and salubrinal on body fat. Dual energy X-ray absorptiometry is used to measure total fat (%) and abdominal fat (%). The asterisks indicate statistical significance at $p < 0.05$ (single), $p < 0.01$ (double) and $p < 0.001$ (triple). (A) Increase in total fat (%) in the OVX mice. (B) Increase in abdominal fat in the OVX mice. (C) Reduction in total fat (%) in the salubrinal treated OVX mice. (D) Decrease in abdominal fat (%) in the salubrinal treated OVX mice. (E) PIXImus images showing the region of abdominal fat measured in the sham OVX, OVX, and salubrinal treated OVX mice.

FIG. 4. Effects of ovariectomy and salubrinal on BMD and BMC. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively. (A) Reduction in BMD (% loss) in total body, lumbar spine, femur, and tibia in the OVX mice in 4 weeks after ovariectomy. (B) Reduction in BMC (% loss) in total body, lumbar spine, femur, and tibia in the OVX mice in 4 weeks after ovariectomy. (C) Increase in BMD (g/cm^2) in lumbar spine in 4 weeks in the salubrinal treated OVX mice. (D) Increase in BMC (g) in lumbar spine in 4 weeks in the salubrinal treated OVX mice.

FIG. 5. Suppression of osteoclast development by subcutaneous administration of salubrinal. The ratios of osteoclast areas are compared among 3 groups (sham OVX, OVX, and salubrinal treated OVX). Data are collected 8 weeks after ovariectomy with and without subcutaneous administration of salubrinal, and shown with mean ± SEM representing nine images (3 independent experiments and 3 images per experiment). The OVX mice show a

significant increase in the osteoclast area compared to the sham OVX mice, while the salubrinal treated OVX mice significantly reduced its area compared to the sham OVX and OVX mice. The microphotographs represent the three groups of osteoclast cultures with TRACP staining. Note that $p < 0.001$ for the salubrinal treated OVX mice vs. the OVX mice, and the OVX mice vs. the sham OVX mice (ANOVA followed by post-hoc *t*-test). Bar = 200 μm.

FIG. 6. Effects of post administration of salubrinal in culture on osteoclast formation. Salubrinal is administered at 3 dosages (1, 2, and 5 μM). The single and triple asterisks indicate $p < 0.05$ and $p < 0.001$, respectively. Bar = 200 μm. (A) Comparison of osteoclast formation in the sham OVX mice with and without post administration of salubrinal in the isolated bone marrow cells. On the left panel, salubrinal was administered from day 0 (initiation of osteoclast culture), while on the right panel from day 4. Salubrinal reduced osteoclast area (%) in both groups in a dosage-dependent manner. The images on the bottom display the representative states of osteoclasts. (B) Comparison of osteoclast formation in the OVX mice with and without post administration of salubrinal in the isolated bone marrow cells. Salubrinal was administered from day 0 on the left panel and from day 4 on the right panel. Salubrinal reduced osteoclast area (%) in a dosage-dependent manner. Four pairs of images on the bottom represent the osteoclast states at 4 different dosages including vehicle control.

FIG. 7. Effects of salubrinal on migration and adhesion of osteoclasts. (A) Haptotaxis of preosteoclasts isolated from the sham OVX, OVX, and salubrinal treated OVX mice. Bone marrow cells were isolated 8 weeks after ovariectomy. Salubrinal or vehicle was administered in the last 4 weeks. Quantitative evaluation of migration in response to M-CSF is performed in the presence and absence of post administration of salubrinal using a transwell assay. Cells isolated from the OVX mice were more active in migration than those from the sham OVX mice, and 4-week salubrinal treatment reduced migration compared to the OVX mice. The images on the bottom display 3 pairs of osteoclast cultures. Date represents mean ± SEM of 10 measurements in each of three independent experiments. Note that *** for $p < 0.001$ between sham OVX and OVX, and OVX and salubrinal treated OVX by ANOVA followed by post-hoc *t*-test. (B) Quantitative evaluation of M-CSF mediated preosteoclast adhesion (30 min) to $\alpha_V\beta_3$. The OVX mice presented greater adhesion that the sham OVX mice, and 4-week salubrinal treatment reduced adhesion compared to the OVX mice. Post administration of salubrinal reduced adhesion in all three groups (sham OVX, OVX, and salubrinal treated OVX). The images on the bottom display 3 pairs of osteoclast cells with and without post-salubrinal treatment. The results are a summary of 8 measurements in each of the three independent experiments. Date represents mean ± SEM. Bar = 200 μm. The asterisks show statistical significance at $p < 0.05$ (single), $p < 0.01$ (double), and $p < 0.001$ (triple),

respectively. Note that *$p < 0.05$ between sham OVX and OVX, and ** $p < 0.01$ between OVX and salubrinal treated OVX as assessed by ANOVA followed by post-hoc *t*-test.

FIG. 8. Effects of salubrinal on colony-forming unit-macrophage/monocyte (CFU-M) of BMMNCs. (A) Salubrinal-induced reduction in CFU-M numbers in the sham OVX mice. Salubrinal at 1, 2, and 5 μM or vehicle was post-administered in cells isolated from three groups of mice. A dosage-dependent change in the CFU-M number was observed. The images on the bottom exhibit the 4 different CFU-M culture conditions, in which the circles indicate the colonies of CFU-M. (B) Comparison of CFU-M numbers among three groups of mice (sham OVX, OVX, and salubrinal treated OVX mice) with and without post administration of salubrinal. Without post-administration of salubrinal the OVX mice presented a larger number of CFU-M colonies than the sham OVX mice, while 4-week salubrinal treatment reduced the CFU-M numbers. The post salubrinal treatment in culture for 7 days reduced the numbers of colonies in all three groups. The representative microphotographs are shown, displaying 6 different conditions in CFU-M cultures with colonies in circle. The results are a summary of three independent experiments. The triple asterisk indicates $p < 0.001$. Bar = 100 μm. Date represents mean ± SEM of ten separate measurements in each of the three experiments. Note that ***$p < 0.001$ between the sham OVX and OVX, and the OVX and pre-salubrinal treated OVX as assessed by ANOVA followed by post-hoc *t*-test.

FIG. 9. Effect of ovariectomy and salubrinal on osteoblast differentiation. ALP staining is used to analyze ALP activity and the percent of ALP positive is calculated. (A) Comparison of CFU-OBL using MSCs isolated from 3 groups of mice (sham OVX, OVX, and salubrinal treated OVX) in the osteoblast differentiating medium. The result shows that 4-week salubrinal treatment increased CFU-OBL (%ALP positive) compared to both the sham OVX and OVX mice. The representative photographs are shown on the right panel. Date represents mean ± SEM of ten measurements in each of the three independent experiments. Note that ***$p < 0.001$ between the salubrinal treated OVX mice and both sham OVX and OVX mice. (B) Effect of post-administration of salubrinal (0.5 μM) on osteoblast differentiation for MSCs isolated 2, 4, and 4 weeks after ovariectomy. Post-salubrinal administration enhanced CFU-OBL (percent of ALP positive) in all three groups. The results are a summary of three independent experiments. The asterisks show statistical significance at $p < 0.05$ (single), $p < 0.01$ (double) and $p < 0.001$ (triple), respectively.

FIG. 10. Effect of ovariectomy and salubrinal on colony forming unit-fibroblast (CFU-F). In CFU-F assay, MSCs from three groups of mice (sham OVX, OVX, and psalubrinal treated OVX) were plated at 2 X 10^6/ml in 2 ml of mouse MesenCult +

Supplemental for 2 weeks. MSCs were stained using HEMA3 Stain Set, and the number of colonies was counted. (A) Comparison of CFU-F in 3 groups of mice (sham OVX, OVX, and salubrinal treated OVX). The result shows that salubrinal treatment increased frequency of CFU-F compared to the sham OVX and OVX mice. The representative photographs of CFU-F in triplicates stained with HEMA3 are shown on the right panel. Date represents mean ± SEM of 9 - 12 measurements in each of the three independent experiments. Note that ***$p < 0.01$ between pre-salubrinal treated OVX mice and sham OVX and **$p < 0.01$ between pre-salubrinal treated OVX mice and OVX mice. (B) Effect of post-administration of salubrinal (0.5 μM) on CFU-F for MSCs isolated from 3 groups of mice (sham OVX, OVX, and pre-salubrinal treated OVX). Post-salubrinal administration improved frequency of CFU-F in all three groups, and significance increase was found in OVX mice. The results are a summary of three independent experiments. The asterisks show statistical significance at $p < 0.05$ (single).

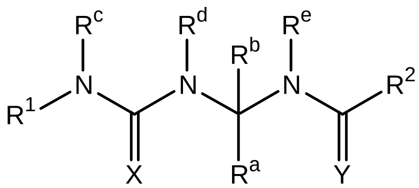
FIG. 11. (A &B) Administration of salubrinal attenuates the RANKL-induced expression of NFATc1 and cathepsin K. (C) Administration of salubrinal elevates the phosphorylation level of EIF2-α (p-eIF2-α) and ATF4 at 24 h. (D) Administration of salubrinal significantly increases osteocalcin (OCN) expression.

FIG. 12. (A) Unloading causes a significant reduction in trabecular bone volume fraction BV/TV (%) in the femur. (B) Administration of salubrinal significantly attenuates the unloading induced loss of BMD in the femur and lumbar spine.

DETAILED DESCRIPTION

Several illustrative embodiments of the invention are described by the following enumerated clauses:

1. A composition for treating a metabolic disorder in a host animal, the composition comprising one or more compounds of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl,

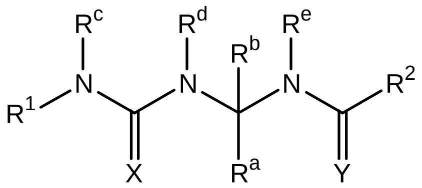
arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

2. A method for treating a metabolic disorder in a host animal, the method comprising the step of administering to the host animal a therapeutically effective amount of one or more compounds of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

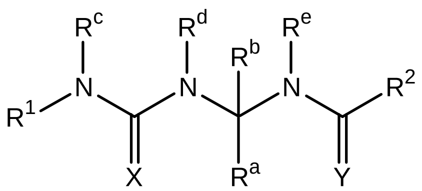
R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo;

or a composition thereof further comprising one or more carriers, diluents, or excipients, or a combination thereof.

3. Use of a therapeutically effective amount of one or more compounds in the manufacture of a medicament for treating a metabolic disorder in a host animal, where the one or more compounds are of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

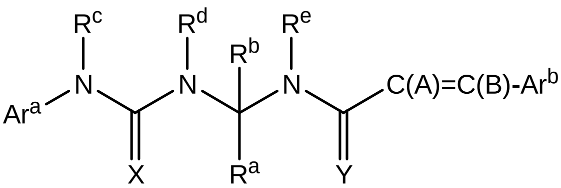
4. The composition or use or method of any one of clauses 1 to 3 wherein R^1 is alkyl, aryl, or heteroaryl, each of which is optionally substituted.

5. The composition or use or method of any one of clauses 1 to 3 wherein R^1 is aryl or heteroaryl, each of which is optionally substituted.

6. The composition or use or method of any one of clauses 1 to 5 wherein R^2 is alkenyl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

7. The composition or use or method of any one of clauses 1 to 5 wherein R^2 is alkenyl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

8. The composition or use or method of any one of clauses 1 to 3 wherein the inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X and Y are independently O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, and a prodrug capable of releasing the attached nitrogen in vivo to form the corresponding H or salt derivative thereof;

and A and B are independently H, or optionally substituted C_1-C_6 alkyl

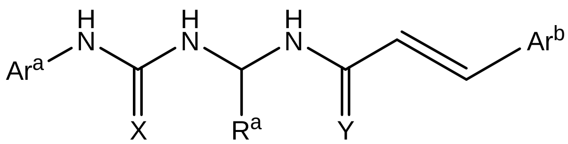
9. The composition or use or method of clause 8 wherein the aryl is a bicyclic aryl.

10. The composition or use or method of clause 8 or 9 wherein the heteroaryl is a bicyclic heteroaryl.

11. The composition or use or method of any one of clauses 11 to 13 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 haloalkyl, C_1-C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

12. The composition or use or method of any one of clauses 8 to 11 wherein the alkenyl has an E geometry.

13. The composition or use or method of any one of clauses 1 to 3 wherein the inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein,

X is O or S;

Y is O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted; and

R^a is optionally substituted alkyl.

14. The composition or use or method of clause 13 wherein the aryl is a bicyclic aryl.

15. The composition or use or method of clause 13 or 14 wherein Ar^a is a bicyclic heteroaryl.

16. The composition or use or method of any one of clauses 13 to 15 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 haloalkyl, C_1-C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused

heterocyclyl.

17. The composition or use or method of any one of clauses 13 to 16 wherein R^a is haloalkyl.

18. The composition or use or method of any one of clauses 13 to 17 wherein R^a is not trifluoromethyl.

19. The composition or use or method of any one of clauses 13 to 18 wherein R^a is haloalkyl, where halo is selected from the group consisting of chloro and bromo, and combinations thereof.

20. The composition or use or method of any one of clauses 13 to 19 wherein R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, and acyl.

21. The composition or use or method of any one of clauses 1 to 3 wherein the inhibitor is salubrinal, or an analog or a derivative thereof, or a pharmaceutically acceptable salt of the foregoing.

22. The composition or use or method of any one of clauses 1 to 21 wherein the medicament further comprises one or more carriers, diluents, or excipients, or a combination thereof.

23. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is osteoporosis.

24. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is osteopenia.

25. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is bone remodeling dysfunction.

26. The composition or use or method of any one of the preceding clauses wherein the composition is capable of increasing BMD, BMC, or a combination thereof in the host animal.

27. The composition or use or method of any one of the preceding clauses wherein the composition is capable of increasing BMD, BMC, or a combination thereof in the lumbar spine host animal.

28. The composition or use or method of any one of the preceding clauses wherein the composition is capable of increasing osteoblast differentiation.

29. The composition or use or method of any one of the preceding clauses wherein the composition is capable of increasing osteoblast function.

30. The composition or use or method of any one of the preceding clauses wherein the composition is capable of decreasing osteoclast differentiation.

31. The composition or use or method of any one of the preceding clauses wherein the composition is capable of decreasing osteoclast function.

32. The composition or use or method of any one of the preceding clauses wherein the composition is capable of increasing CFU-OBL in the host animal.

33. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is a fat metabolism dysfunction.

34. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is a fat storage dysfunction.

35. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder is obesity.

36. The composition or use or method of any one of the preceding clauses wherein the composition is capable of maintaining or decreasing fat load in the host animal.

37. The composition or use or method of any one of the preceding clauses wherein the composition is capable of maintaining or decreasing abdominal fat load in the host animal.

38. The composition or use or method of any one of the preceding clauses wherein the composition is capable of decreasing adipocyte differentiation.

39. The composition or use or method of any one of the preceding clauses wherein the composition is capable of decreasing adipocyte cell division.

40. The composition or use or method of any one of the preceding clauses wherein the metabolic disorder results in dysuria

41. The composition or use or method of clause 40 wherein the dysuria is urinary urgency.

42. The composition or use or method of clause 40 or 41 wherein the dysuria is urinary frequency.

43. The composition or use or method of any one of the preceding clauses wherein the host animal is female.

44. The composition or use or method of clause 43 wherein the metabolic disorder is post-menopausal osteoporosis.

45. The composition or use or method of clause 43 or 44 wherein the metabolic disorder is post-menopausal osteopenia.

46. The composition or use or method of any one of clauses 43 to 45 wherein the metabolic disorder is post-menopausal obesity.

47. The composition or use or method of any one of clauses 43 to 46 wherein the composition is capable of maintaining or increasing uterine weight load.

48. The composition or use or method of any one of clauses 1 to 47 wherein the host animal is a human.

In another embodiment, compositions described herein, including those containing salubrinal (480 Da, $C_{21}H_{17}Cl_3N_4OS$), block de-phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) (ref). The elevated level of the phosphorylated form of eIF2a reduces translation efficiency except for particular proteins such as activating transcription factor 4 (ATF4). In another embodiment, compositions described herein, including those containing salubrinal, decrease NFATc1 expression, including RANKL-induced NFATc1 expression. In another embodiment, compositions described herein, including those containing salubrinal, decrease cathepsin K expression, including RANKL-induced cathepsin K expression. In another embodiment, compositions described herein, including those containing salubrinal, decrease OCN expression.

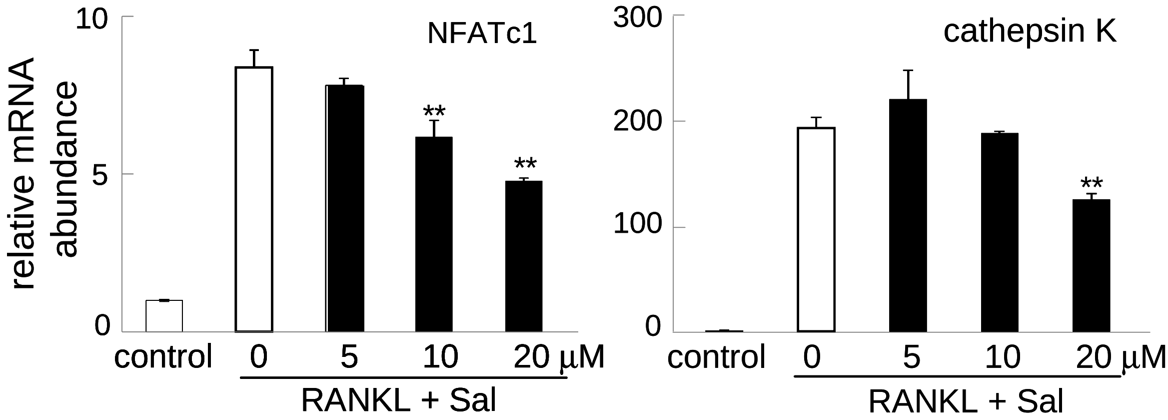


FIG. 11A

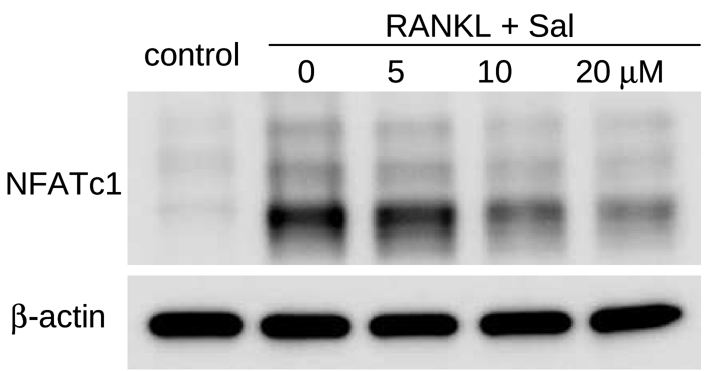


FIG. 11B

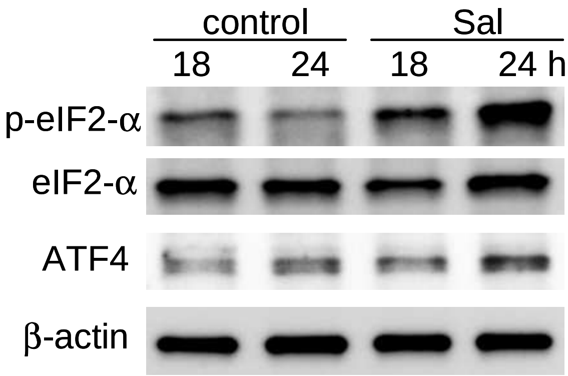


FIG. 11C

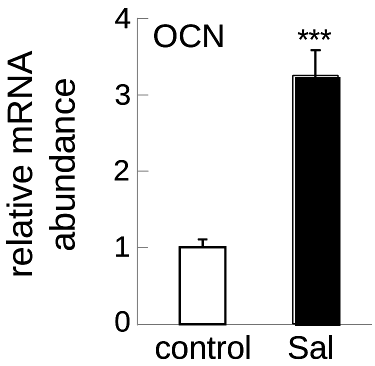


FIG. 11D

In each of the foregoing and following embodiments, it is to be understood that the formulae include and represent not only all pharmaceutically acceptable salts of the compounds, but also include any and all hydrates and/or solvates of the compound formulae. It is appreciated that certain functional groups, such as the hydroxy, amino, and like groups form complexes and/or coordination compounds with water and/or various solvents, in the various physical forms of the compounds. Accordingly, the above formulae are to be understood to include and represent those various hydrates and/or solvates. In each of the foregoing and following embodiments, it is also to be understood that the formulae include and represent each possible isomer, such as stereoisomers and geometric isomers, both individually and in any and all possible mixtures. In each of the foregoing and following embodiments, it is also to be understood that the formulae include and represent any and all crystalline forms, partially crystalline forms, and non crystalline and/or amorphous forms of the compounds.

Illustrative derivatives include, but are not limited to, both those compounds that may be synthetically prepared from the compounds described herein, as well as those compounds that may be prepared in a similar way as those described herein, but differing in the selection of starting materials. It is to be understood that such derivatives may include prodrugs of the compounds described herein, compounds described herein that include one or more protection or protecting groups, including compounds that are used in the preparation of other compounds described herein.

The compounds described herein may contain one or more chiral centers, or may otherwise be capable of existing as multiple stereoisomers. It is to be understood that in one embodiment, the invention described herein is not limited to any particular sterochemical requirement, and that the compounds, and compositions, methods, uses, and medicaments that include them may be optically pure, or may be any of a variety of stereoisomeric mixtures, including racemic and other mixtures of enantiomers, other mixtures of diastereomers, and the like. It is also to be understood that such mixtures of stereoisomers may include a single

stereochemical configuration at one or more chiral centers, while including mixtures of stereochemical configuration at one or more other chiral centers.

Similarly, the compounds described herein may be include geometric centers, such as cis, trans, E, and Z double bonds. It is to be understood that in another embodiment, the invention described herein is not limited to any particular geometric isomer requirement, and that the compounds, and compositions, methods, uses, and medicaments that include them may be pure, or may be any of a variety of geometric isomer mixtures. It is also to be understood that such mixtures of geometric isomers may include a single configuration at one or more double bonds, while including mixtures of geometry at one or more other double bonds.

As used herein, the term “alkyl” includes a chain of carbon atoms, which is optionally branched. As used herein, the term “alkenyl” and “alkynyl” includes a chain of carbon atoms, which is optionally branched, and includes at least one double bond or triple bond, respectively. It is to be understood that alkynyl may also include one or more double bonds. It is to be further understood that in certain embodiments, alkyl is advantageously of limited length, including C_1-C_{24}, C_1-C_{12}, C_1-C_8, C_1-C_6, and C_1-C_4. Illustratively, such particularly limited length alkyl groups, including C_1-C_8, C_1-C_6, and C_1-C_4 may be referred to as lower alkyl. It is to be further understood that in certain embodiments alkenyl and/or alkynyl may each be advantageously of limited length, including C_2-C_{24}, C_2-C_{12}, C_2-C_8, C_2-C_6, and C_2-C_4. Illustratively, such particularly limited length alkenyl and/or alkynyl groups, including C_2-C_8, C_2-C_6, and C_2-C_4 may be referred to as lower alkenyl and/or alkynyl. It is appreciated herein that shorter alkyl, alkenyl, and/or alkynyl groups may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkyl refers to alkyl as defined herein, and optionally lower alkyl. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkenyl refers to alkenyl as defined herein, and optionally lower alkenyl. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkynyl refers to alkynyl as defined herein, and optionally lower alkynyl. Illustrative alkyl, alkenyl, and alkynyl groups are, but not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, 2-pentyl, 3-pentyl, neopentyl, hexyl, heptyl, octyl, and the like, and the corresponding groups containing one or more double and/or triple bonds, or a combination thereof.

As used herein, the term “alkylene” includes a divalent chain of carbon atoms, which is optionally branched. As used herein, the term “alkenylene” and “alkynylene” includes a divalent chain of carbon atoms, which is optionally branched, and includes at least one double

bond or triple bond, respectively. It is to be understood that alkynylene may also include one or more double bonds. It is to be further understood that in certain embodiments, alkylene is advantageously of limited length, including C_1-C_{24}, C_1-C_{12}, C_1-C_8, C_1-C_6, and C_1-C_4. Illustratively, such particularly limited length alkylene groups, including C_1-C_8, C_1-C_6, and C_1-C_4 may be referred to as lower alkylene. It is to be further understood that in certain embodiments alkenylene and/or alkynylene may each be advantageously of limited length, including C_2-C_{24}, C_2-C_{12}, C_2-C_8, C_2-C_6, and C_2-C_4. Illustratively, such particularly limited length alkenylene and/or alkynylene groups, including C_2-C_8, C_2-C_6, and C_2-C_4 may be referred to as lower alkenylene and/or alkynylene. It is appreciated herein that shorter alkylene, alkenylene, and/or alkynylene groups may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkylene, alkenylene, and alkynylene refers to alkylene, alkenylene, and alkynylene as defined herein, and optionally lower alkylene, alkenylene, and alkynylene. Illustrative alkyl groups are, but not limited to, methylene, ethylene, n-propylene, isopropylene, n-butylene, isobutylene, sec-butylene, pentylene, 1,2-pentylene, 1,3-pentylene, hexylene, heptylene, octylene, and the like.

As used herein, the term "cycloalkyl" includes a chain of carbon atoms, which is optionally branched, where at least a portion of the chain in cyclic. It is to be understood that cycloalkylalkyl is a subset of cycloalkyl. It is to be understood that cycloalkyl may be polycyclic. Illustrative cycloalkyl include, but are not limited to, cyclopropyl, cyclopentyl, cyclohexyl, 2-methylcyclopropyl, cyclopentyleth-2-yl, adamantyl, and the like. As used herein, the term "cycloalkenyl" includes a chain of carbon atoms, which is optionally branched, and includes at least one double bond, where at least a portion of the chain in cyclic. It is to be understood that the one or more double bonds may be in the cyclic portion of cycloalkenyl and/or the non-cyclic portion of cycloalkenyl. It is to be understood that cycloalkenylalkyl and cycloalkylalkenyl are each subsets of cycloalkenyl. It is to be understood that cycloalkyl may be polycyclic. Illustrative cycloalkenyl include, but are not limited to, cyclopentenyl, cyclohexylethen-2-yl, cycloheptenylpropenyl, and the like. It is to be further understood that chain forming cycloalkyl and/or cycloalkenyl is advantageously of limited length, including C_3-C_{24}, C_3-C_{12}, C_3-C_8, C_3-C_6, and C_5-C_6. It is appreciated herein that shorter alkyl and/or alkenyl chains forming cycloalkyl and/or cycloalkenyl, respectively, may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior.

As used herein, the term "heteroalkyl" includes a chain of atoms that includes both carbon and at least one heteroatom, and is optionally branched. Illustrative heteroatoms include nitrogen, oxygen, and sulfur. In certain variations, illustrative heteroatoms also include

phosphorus, and selenium. As used herein, the term “cycloheteroalkyl” including heterocyclyl and heterocycle, includes a chain of atoms that includes both carbon and at least one heteroatom, such as heteroalkyl, and is optionally branched, where at least a portion of the chain is cyclic. Illustrative heteroatoms include nitrogen, oxygen, and sulfur. In certain variations, illustrative heteroatoms also include phosphorus, and selenium. Illustrative cycloheteroalkyl include, but are not limited to, tetrahydrofuryl, pyrrolidinyl, tetrahydropyranyl, piperidinyl, morpholinyl, piperazinyl, homopiperazinyl, quinuclidinyl, and the like.

As used herein, the term “aryl” includes monocyclic and polycyclic aromatic carbocyclic groups, each of which may be optionally substituted. Illustrative aromatic carbocyclic groups described herein include, but are not limited to, phenyl, naphthyl, and the like. As used herein, the term “heteroaryl” includes aromatic heterocyclic groups, each of which may be optionally substituted. Illustrative aromatic heterocyclic groups include, but are not limited to, pyridinyl, pyrimidinyl, pyrazinyl, triazinyl, tetrazinyl, quinolinyl, quinazolinyl, quinoxalinyl, thienyl, pyrazolyl, imidazolyl, oxazolyl, thiazolyl, isoxazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, benzimidazolyl, benzoxazolyl, benzthiazolyl, benzisoxazolyl, benzisothiazolyl, and the like.

As used herein, the term “amino” includes the group NH_2, alkylamino, and dialkylamino, where the two alkyl groups in dialkylamino may be the same or different, i.e. alkylalkylamino. Illustratively, amino includes methylamino, ethylamino, dimethylamino, methylethylamino, and the like. In addition, it is to be understood that when amino modifies or is modified by another term, such as aminoalkyl, or acylamino, the above variations of the term amino are included therein. Illustratively, aminoalkyl includes H_2N-alkyl, methylaminoalkyl, ethylaminoalkyl, dimethylaminoalkyl, methylethylaminoalkyl, and the like. Illustratively, acylamino includes acylmethylamino, acylethylamino, and the like.

As used herein, the term “amino and derivatives thereof” includes amino as described herein, and alkylamino, alkenylamino, alkynylamino, heteroalkylamino, heteroalkenylamino, heteroalkynylamino, cycloalkylamino, cycloalkenylamino, cycloheteroalkylamino, cycloheteroalkenylamino, arylamino, arylalkylamino, arylalkenylamino, arylalkynylamino, heteroarylamino, heteroarylalkylamino, heteroarylalkenylamino, heteroarylalkynylamino, acylamino, and the like, each of which is optionally substituted. The term “amino derivative” also includes urea, carbamate, and the like.

As used herein, the term “hydroxy and derivatives thereof” includes OH, and alkyloxy, alkenyloxy, alkynyloxy, heteroalkyloxy, heteroalkenyloxy, heteroalkynyloxy, cycloalkyloxy, cycloalkenyloxy, cycloheteroalkyloxy, cycloheteroalkenyloxy, aryloxy, arylalkyloxy, arylalkenyloxy, arylalkynyloxy, heteroaryloxy, heteroarylalkyloxy,

heteroarylalkenyloxy, heteroarylalkynyloxy, acyloxy, and the like, each of which is optionally substituted. The term “hydroxy derivative” also includes carbamate, and the like.

As used herein, the term “thio and derivatives thereof” includes SH, and alkylthio, alkenylthio, alkynylthio, heteroalkylthio, heteroalkenylthio, heteroalkynylthio, cycloalkylthio, cycloalkenylthio, cycloheteroalkylthio, cycloheteroalkenylthio, arylthio, arylalkylthio, arylalkenylthio, arylalkynylthio, heteroarylthio, heteroarylalkylthio, heteroarylalkenylthio, heteroarylalkynylthio, acylthio, and the like, each of which is optionally substituted. The term “thio derivative” also includes thiocarbamate, and the like.

As used herein, the term “acyl” includes formyl, and alkylcarbonyl, alkenylcarbonyl, alkynylcarbonyl, heteroalkylcarbonyl, heteroalkenylcarbonyl, heteroalkynylcarbonyl, cycloalkylcarbonyl, cycloalkenylcarbonyl, cycloheteroalkylcarbonyl, cycloheteroalkenylcarbonyl, arylcarbonyl, arylalkylcarbonyl, arylalkenylcarbonyl, arylalkynylcarbonyl, heteroarylcarbonyl, heteroarylalkylcarbonyl, heteroarylalkenylcarbonyl, heteroarylalkynylcarbonyl, acylcarbonyl, and the like, each of which is optionally substituted.

As used herein, the term “carbonyl and derivatives thereof” includes the group C(O), C(S), C(NH) and substituted amino derivatives thereof.

As used herein, the term “carboxylic acid and derivatives thereof” includes the group CO_2H and salts thereof, and esters and amides thereof, and CN.

As used herein, the term “sulfonic acid or a derivative thereof” includes SO_3H and salts thereof, and esters and amides thereof.

As used herein, the term “sulfonyl” includes alkylsulfonyl, alkenylsulfonyl, alkynylsulfonyl, heteroalkylsulfonyl, heteroalkenylsulfonyl, heteroalkynylsulfonyl, cycloalkylsulfonyl, cycloalkenylsulfonyl, cycloheteroalkylsulfonyl, cycloheteroalkenylsulfonyl, arylsulfonyl, arylalkylsulfonyl, arylalkenylsulfonyl, arylalkynylsulfonyl, heteroarylsulfonyl, heteroarylalkylsulfonyl, heteroarylalkenylsulfonyl, heteroarylalkynylsulfonyl, acylsulfonyl, and the like, each of which is optionally substituted.

The term "optionally substituted" as used herein includes the replacement of hydrogen atoms with other functional groups on the radical that is optionally substituted. Such other functional groups illustratively include, but are not limited to, amino, hydroxyl, halo, thiol, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, nitro, sulfonic acids and derivatives thereof, carboxylic acids and derivatives thereof, and the like. Illustratively, any of amino, hydroxyl, thiol, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, and/or sulfonic acid is optionally substituted.

As used herein, the terms "optionally substituted aryl" and "optionally

substituted heteroaryl" include the replacement of hydrogen atoms with other functional groups on the aryl or heteroaryl that is optionally substituted. Such other functional groups illustratively include, but are not limited to, amino, hydroxy, halo, thio, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, nitro, sulfonic acids and derivatives thereof, carboxylic acids and derivatives thereof, and the like. Illustratively, any of amino, hydroxy, thio, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, and/or sulfonic acid is optionally substituted.

Illustrative substituents include, but are not limited to, a radical $-(CH_2)_xZ^X$, where x is an integer from 0-6 and Z^X is selected from halogen, hydroxy, alkanoyloxy, including C_1-C_6 alkanoyloxy, optionally substituted aroyloxy, alkyl, including C_1-C_6 alkyl, alkoxy, including C_1-C_6 alkoxy, cycloalkyl, including C_3-C_8 cycloalkyl, cycloalkoxy, including C_3-C_8 cycloalkoxy, alkenyl, including C_2-C_6 alkenyl, alkynyl, including C_2-C_6 alkynyl, haloalkyl, including C_1-C_6 haloalkyl, haloalkoxy, including C_1-C_6 haloalkoxy, halocycloalkyl, including C_3-C_8 halocycloalkyl, halocycloalkoxy, including C_3-C_8 halocycloalkoxy, amino, C_1-C_6 alkylamino, (C_1-C_6 alkyl)(C_1-C_6 alkyl)amino, alkylcarbonylamino, N-(C_1-C_6 alkyl)alkylcarbonylamino, aminoalkyl, C_1-C_6 alkylaminoalkyl, (C_1-C_6 alkyl)(C_1-C_6 alkyl)aminoalkyl, alkylcarbonylaminoalkyl, N-(C_1-C_6 alkyl)alkylcarbonylaminoalkyl, cyano, and nitro; or Z^X is selected from $-CO_2R^4$ and $-CONR^5R^6$, where R^4, R^5, and R^6 are each independently selected in each occurrence from hydrogen, C_1-C_6 alkyl, aryl-C_1-C_6 alkyl, and heteroaryl-C_1-C_6 alkyl.

The term “prodrug” as used herein generally refers to any compound that when administered to a biological system generates a biologically active compound as a result of one or more spontaneous chemical reaction(s), enzyme-catalyzed chemical reaction(s), and/or metabolic chemical reaction(s), or a combination thereof. In vivo, the prodrug is typically acted upon by an enzyme (such as esterases, amidases, phosphatases, and the like), simple biological chemistry, or other process in vivo to liberate or regenerate the more pharmacologically active drug. This activation may occur through the action of an endogenous host enzyme or a non-endogenous enzyme that is administered to the host preceding, following, or during administration of the prodrug. Additional details of prodrug use are described in U.S. Pat. No. 5,627,165; and Pathalk et al., Enzymic protecting group techniques in organic synthesis, Stereosel. Biocatal. 775-797 (2000). It is appreciated that the prodrug is advantageously converted to the original drug as soon as the goal, such as targeted delivery, safety, stability, and the like is achieved, followed by the subsequent rapid elimination of the released remains of the group forming the prodrug.

Prodrugs may be prepared from the compounds described herein by attaching groups that ultimately cleave in vivo to one or more functional groups present on the compound, such as -OH-, -SH, -CO_2H, -NR_2. Illustrative prodrugs include but are not limited to carboxylate esters where the group is alkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, acyloxyalkyl, alkoxycarbonyloxyalkyl as well as esters of hydroxyl, thiol and amines where the group attached is an acyl group, an alkoxycarbonyl, aminocarbonyl, phosphate or sulfate. Illustrative esters, also referred to as active esters, include but are not limited to 1-indanyl, N-oxysuccinimide; acyloxyalkyl groups such as acetoxymethyl, pivaloyloxymethyl, β-acetoxyethyl, β-pivaloyloxyethyl, 1-(cyclohexylcarbonyloxy)prop-1-yl, (1 -aminoethyl)carbonyloxymethyl, and the like; alkoxycarbonyloxyalkyl groups, such as ethoxycarbonyloxymethyl, α-ethoxycarbonyloxyethyl, β-ethoxycarbonyloxyethyl, and the like; dialkylaminoalkyl groups, including di-lower alkylamino alkyl groups, such as dimethylaminomethyl, dimethylaminoethyl, diethylaminomethyl, diethylaminoethyl, and the like; 2-(alkoxycarbonyl)-2-alkenyl groups such as 2-(isobutoxycarbonyl) pent-2-enyl, 2-(ethoxycarbonyl)but-2-enyl, and the like; and lactone groups such as phthalidyl, dimethoxyphthalidyl, and the like.

Further illustrative prodrugs contain a chemical moiety, such as an amide or phosphorus group functioning to increase solubility and/or stability of the compounds described herein. Further illustrative prodrugs for amino groups include, but are not limited to, (C_3-C_{20})alkanoyl; halo-(C_3-C_{20})alkanoyl; (C_3-C_{20})alkenoyl; (C_4-C_7)cycloalkanoyl; (C_3-C_6)-cycloalkyl(C_2-C_{16})alkanoyl; optionally substituted aroyl, such as unsubstituted aroyl or aroyl substituted by 1 to 3 substituents selected from the group consisting of halogen, cyano, trifluoromethanesulphonyloxy, (C_1-C_3)alkyl and (C_1-C_3)alkoxy, each of which is optionally further substituted with one or more of 1 to 3 halogen atoms; optionally substituted aryl(C_2-C_{16})alkanoyl and optionally substituted heteroaryl(C_2-C_{16})alkanoyl, such as the aryl or heteroaryl radical being unsubstituted or substituted by 1 to 3 substituents selected from the group consisting of halogen, (C_1-C_3)alkyl and (C_1-C_3)alkoxy, each of which is optionally further substituted with 1 to 3 halogen atoms; and optionally substituted heteroarylalkanoyl having one to three heteroatoms selected from O, S and N in the heteroaryl moiety and 2 to 10 carbon atoms in the alkanoyl moiety, such as the heteroaryl radical being unsubstituted or substituted by 1 to 3 substituents selected from the group consisting of halogen, cyano, trifluoromethanesulphonyloxy, (C_1-C_3)alkyl, and (C_1-C_3)alkoxy, each of which is optionally further substituted with 1 to 3 halogen atoms. The groups illustrated are exemplary, not exhaustive, and may be prepared by conventional processes.

It is understood that the prodrugs themselves may not possess significant

biological activity, but instead undergo one or more spontaneous chemical reaction(s), enzyme-catalyzed chemical reaction(s), and/or metabolic chemical reaction(s), or a combination thereof after administration in vivo to produce the compound described herein that is biologically active or is a precursor of the biologically active compound. However, it is appreciated that in some cases, the prodrug is biologically active. It is also appreciated that prodrugs may often serves to improve drug efficacy or safety through improved oral bioavailability, pharmacodynamic half-life, and the like. Prodrugs also refer to derivatives of the compounds described herein that include groups that simply mask undesirable drug properties or improve drug delivery. For example, one or more compounds described herein may exhibit an undesirable property that is advantageously blocked or minimized may become pharmacological, pharmaceutical, or pharmacokinetic barriers in clinical drug application, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity, and poor patient acceptance (bad taste, odor, pain at injection site, and the like), and others. It is appreciated herein that a prodrug, or other strategy using reversible derivatives, can be useful in the optimization of the clinical application of a drug.

As used herein, the term “composition” generally refers to any product comprising the specified ingredients in the specified amounts, as well as any product which results, directly or indirectly, from combinations of the specified ingredients in the specified amounts. It is to be understood that the compositions described herein may be prepared from isolated compounds described herein or from salts, solutions, hydrates, solvates, and other forms of the compounds described herein. It is also to be understood that the compositions may be prepared from various amorphous, non-amorphous, partially crystalline, crystalline, and/or other morphological forms of the compounds described herein. It is also to be understood that the compositions may be prepared from various hydrates and/or solvates of the compounds described herein. Accordingly, such pharmaceutical compositions that recite compounds described herein are to be understood to include each of, or any combination of, the various morphological forms and/or solvate or hydrate forms of the compounds described herein. Illustratively, compositions may include one or more carriers, diluents, and/or excipients. The compounds described herein, or compositions containing them, may be formulated in a therapeutically effective amount in any conventional dosage forms appropriate for the methods described herein. The compounds described herein, or compositions containing them, including such formulations, may be administered by a wide variety of conventional routes for the methods described herein, and in a wide variety of dosage formats, utilizing known procedures (see generally, Remington: The Science and Practice of Pharmacy, (21st ed., 2005)).

The term “therapeutically effective amount” as used herein, refers to that amount

of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated. In one aspect, the therapeutically effective amount is that which may treat or alleviate the disease or symptoms of the disease at a reasonable benefit/risk ratio applicable to any medical treatment. However, it is to be understood that the total daily usage of the compounds and compositions described herein may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically-effective dose level for any particular patient will depend upon a variety of factors, including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, gender and diet of the patient: the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidentally with the specific compound employed; and like factors well known to the researcher, veterinarian, medical doctor or other clinician of ordinary skill.

In addition to the illustrative dosages and dosing protocols described herein, it is to be understood that an effective amount of any one or a mixture of the compounds described herein can be readily determined by the attending diagnostician or physician by the use of known techniques and/or by observing results obtained under analogous circumstances. In determining the effective amount or dose, a number of factors are considered by the attending diagnostician or physician, including, but not limited to the species of mammal, including human, its size, age, and general health, the specific disease or disorder involved, the degree of or involvement or the severity of the disease or disorder, the response of the individual patient, the particular compound administered, the mode of administration, the bioavailability characteristics of the preparation administered, the dose regimen selected, the use of concomitant medication, and other relevant circumstances.

The dosage of each compound of the claimed combinations depends on several factors, including: the administration method, the condition to be treated, the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the person to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular patient may affect the dosage used.

It is to be understood that in the methods described herein, the individual components of a co-administration, or combination can be administered by any suitable means, contemporaneously, simultaneously, sequentially, separately or in a single pharmaceutical

formulation. Where the co-administered compounds or compositions are administered in separate dosage forms, the number of dosages administered per day for each compound may be the same or different. The compounds or compositions may be administered via the same or different routes of administration. The compounds or compositions may be administered according to simultaneous or alternating regimens, at the same or different times during the course of the therapy, concurrently in divided or single forms.

The term “administering” as used herein includes all means of introducing the compounds and compositions described herein to the patient, including, but are not limited to, oral (po), intravenous (iv), intramuscular (im), subcutaneous (sc), transdermal, inhalation, buccal, ocular, sublingual, vaginal, rectal, and the like. The compounds and compositions described herein may be administered in unit dosage forms and/or formulations containing conventional nontoxic pharmaceutically-acceptable carriers, adjuvants, and vehicles.

Illustrative formats for oral administration include tablets, capsules, elixirs, syrups, and the like.

Illustrative routes for parenteral administration include intravenous, intraarterial, intraperitoneal, epidurial, intraurethral, intrasternal, intramuscular and subcutaneous, as well as any other art recognized route of parenteral administration.

Illustratively, administering includes local use, such as when administered locally to the site of disease, injury, or defect, or to a particular organ or tissue system. Illustrative local administration may be performed during open surgery, or other procedures when the site of disease, injury, or defect is accessible. Alternatively, local administration may be performed using parenteral delivery where the compound or compositions described herein are deposited locally to the site without general distribution to multiple other non-target sites in the patient being treated. It is further appreciated that local administration may be directly in the injury site, or locally in the surrounding tissue. Similar variations regarding local delivery to particular tissue types, such as organs, and the like, are also described herein. Illustratively, compounds may be administered directly to the nervous system including, but not limited to, intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration by delivery via intracranial or intravertebral needles and/or catheters with or without pump devices.

Depending upon the disease as described herein, the route of administration and/or whether the compounds and/or compositions are administered locally or systemically, a wide range of permissible dosages are contemplated herein, including doses falling in the range from about 1 μg/kg to about 1 g/kg. The dosages may be single or divided, and may administered according to a wide variety of protocols, including q.d., b.i.d., t.i.d., or even every

other day, once a week, once a month, once a quarter, and the like. In each of these cases it is understood that the therapeutically effective amounts described herein correspond to the instance of administration, or alternatively to the total daily, weekly, month, or quarterly dose, as determined by the dosing protocol.

In making the pharmaceutical compositions of the compounds described herein, a therapeutically effective amount of one or more compounds in any of the various forms described herein may be mixed with one or more excipients, diluted by one or more excipients, or enclosed within such a carrier which can be in the form of a capsule, sachet, paper, or other container. Excipients may serve as a diluent, and can be solid, semi-solid, or liquid materials, which act as a vehicle, carrier or medium for the active ingredient. Thus, the formulation compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders. The compositions may contain anywhere from about 0.1% to about 99.9% active ingredients, depending upon the selected dose and dosage form.

The effective use of the compounds, compositions, and methods described herein for treating or ameliorating one or more effects of metabolic disorder using one or more compounds described herein may be based upon animal models, such as murine, canine, porcine, and non-human primate animal models of disease. For example, it is understood that metabolic disorder in humans may be characterized by a loss of function, and/or the development of symptoms, each of which may be elicited in animals, such as mice, and other surrogate test animals. In particular the OVX mouse model may be used to evaluate the methods of treatment and the pharmaceutical compositions described herein to determine the therapeutically effective amounts described herein.

For example, OVX mice are reported to mimic the acute effects of menopause in mineral and fat metabolism (Sun 2006; Weitzmann 2006). Those mice models present not only bone resorption by increasing osteoclast formation but also an increase in bone formation by osteoblasts (Jilka 1998). In particular, the removal of ovaries is reported to result in an increased commitment of stromal cells to the osteoblastic lineage (Jilka 1998) and enhanced proliferation of early osteoblast precursors (Di 2001), as well as an elevated activity of osteoclasts (Kimble 1996). However, it has also been reported that the overall osteoclast activity is more profound, and results in a net bone loss, and osteoporosis and/or osteopenia (Li et al., "Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand" PNAS 108(2): 768-73 (2011)).

Further, an increase in body weight is often associated with the postmenopausal

condition (Pirro 2010), and there is supporting evidence that osteoporosis and obesity may share common metabolic factors (Rosen 2006). OVX mice also exhibit a weight increase phenotype. In this study, we examined the effects of salubrinal to OVX mice focusing on not only development of osteoclasts and osteoblasts in bone remodeling but also body weight and fat metabolism.

Although compounds described herein may modulate bone remodeling and fat metabolism at various regulatory levels, it has been discovered herein that the compounds also show specific effects on bone marrow derived cells and their developmental fates. Bone marrow is the source of mesenchymal stem cells (MSCs) (Mosna 2010), which give rise to osteoblasts and adipocytes (Cao 2012; Herhi 2012), as well as osteoclasts that have a myeloid monocyte/macrophage lineage (Boyce 2009). Osteoblasts contribute to forming new skeletal matrix (Tare 2010), while osteoclasts adhere to the matrix and release bone resorbing factors. Bone resorption by osteoclasts increases in pathologic states such as in OVX mice (Henrikasen 2007). It is reported that OVX mice 8 weeks after ovariectomy show bone loss accompanied with the activation of osteoclastogenesis (Pierroz 2009). It has been discovered herein that the compounds described herein are useful in treating bone loss through inhibition of osteoclast differentiation and by stimulation of osteoblast development.

The following examples further illustrate specific embodiments of the invention; however, the following illustrative examples should not be interpreted in any way to limit the invention.

EXAMPLES

METHOD EXAMPLE. Animals and materials preparation. Seventy-two C57BL/6 female mice (~12 weeks of age) are used. Four to five mice are housed per cage and fed with mouse chow and water *ad libitum*. Forty-eight mice are subjected to ovariectomy (OVX), and twenty-four mice are used as sham ovariectomy (age-match sham OVX control). Compounds and reagents are prepared using conventional methods, or purchased from commercial suppliers such as Sigma (St. Louis, MO), cytokines from PeproTech (Rocky Hills, NC), salubrinal from Tocris Bioscience (Ellisville, MO, USA), and the like.

METHOD EXAMPLE. Ovariectomy (OVX). All animals are weighed prior to ovariectomy and at sacrifice (FIG. 1). Anesthesia is conducted with 1.5% isoflurane at a flow rate of 0.5 to 1.0 L/min. Mice are placed in ventral recumbency. The hair at the operative sites (dorsal mid-lumbar area) is shaved, and the skin is cleaned with 70% alcohol and 10% providoneiodine solution. With the scalpel, a 10-mm skin incision is made on the midline of dorsa between the caudal edge of ribcage and the pelvis. For removing a pair of ovaries, the muscle wall is incised and the abdominal cavity is reached. After removing ovaries, the uterus

body is placed back into the abdominal cavity and the wound is closed by suturing. For sham OVX mice, the same procedure was conducted without removing ovaries.

During the 8 weeks of experiment, administration of compounds described herein, including salubrinal, is conducted from week 5. The mice are sacrificed at 2, 4, and 8 weeks after OVX surgery. Uteri are harvested to evaluate the effect of ovariectomy and administration of test compound, such as salubrinal (FIG. 2).

METHOD EXAMPLE. Tail suspension: Mice (male and female) are anesthetized by inhalation of isoflurane, and the tail is inserted into a plastic tube of the tail harness and glued with super glue. The animal is placed head-down at approximately a 30 – 40˚ angle that prohibits the hindlimbs from reaching the ground. The tail harness is attached to a metal swivel (fishing wire) and connected to a metal support that horizontally spans a housing cage from one corner to the other. The mouse is able to move along the horizontal metal support and access about 80% of the cage (Hino et al. 2007; Zhang et al. 2008). Water (hydrogel placed on floor) and chow (placed on floor) is provided ad libitum in a plastic dish throughout the experiment, ca. 2 weeks.

METHOD EXAMPLE. Administration of compounds described herein. Forty-eight mice (12 mice in each group) are subjected to compound treatment. During tail suspension, test compounds, such as salubrinal (1 mg/kg body weight) is administered daily by subcutaneous (SC) injection for a period of four weeks. The control group receives an equal volume of vehicle.

METHOD EXAMPLE. Measurements of bone mineral density (BMD) and bone mineral content (BMC). Using peripheral dual energy X-ray absorptiometry designed for analyzing mouse skeletons (DXA; PIXImus II, Lunar Corp., Madison, WI), BMD and BMC are determined (Zhang 2011). The device is suitable for measurements of various compositions of small animals weighing 10–50 g. In brief, each mouse is anesthetized in an anesthetic induction chamber and then mask-anesthetized using 1.5% isoflurane. The mouse is placed on the PIXImus platform in the prone position on a specimen tray, and an image is acquired in less than 5 min. Whole body bone mineral density (BMD; g/cm^2) and bone mineral content (BMC; mg) is determined without including the head. Furthermore, BMD and BMC of the lumbar spine, femur, and tibia are determined with mouse-specific software (version 1.47) (FIG. 4).

METHOD EXAMPLE. Measurements of total fat and abdominal fat (%). Compositions of body fats such as whole body fat contents (%) and abdominal fat content (%) are measured using dual-energy X-ray absorptiometry images (Kim 2006). Abdominal fat content (%) is assessed in the region of interest, which is defined from the caudal edge of ribcage to the pelvis (~ 100 pixels apart) (FIG. 3).

METHOD EXAMPLE. Isolation of bone marrow mononuclear cells (BMMNCs) and generation of murine osteoclasts. Murine BMMNCs are collected by flushing the iliac, femurs and tibias with Iscove's MEM (Gibco-Invitrogen, Carsbad, USA) containing 2% fetal bovine serum (FBS, Sigma, St. Louis, MO) using a 23-gauge needle. BMMNCs are then separated by low-density gradient centrifugation. BMMNCs are cultured for 3 days in α-MEM supplemented with 10% FBS, 30 ng/ml murine macrophage-colony stimulating factor (M-CSF), and 20 ng/ml murine receptor activator of nuclear factor kappa-B ligand (RANKL). On day 3, culture medium is replaced by α-MEM supplemented with 10% FBS, 30 ng/ml M-CSF, and 60 ng/ml RANKL for additional 3 days. Formation of multi-nucleated osteoclasts may be initiated by osteoclastogenic factors, such as receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Lau 2007; Kong 1999).

METHOD EXAMPLE. Osteoclast differentiation assay. The effect of compounds described herein on osteoclast differentiation is analyzed as a dose response. For example, dosages of salubrinal (0, 1, 2, and 5 μM) were evaluated. An osteoclast differentiation assay is carried out in 96-well plates (FIG. 5 and FIG. 6).

In a first set of experiments, test compound, such as salubrinal, is administered for 7 days, beginning on day 0. In a second set of experiments, test compound, such as salubrinal, is administered for 3 days, beginning on day 4. During 6-day experiments, culture medium is exchanged once on day 4. Cells are fixed and stained with a tartrate resistant acid phosphate (TRACP)-staining kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. TRACP-positive multinuclear cells (> 3 nuclei) are identified as osteoclasts and their numbers are counted under an inverted microscope. Each osteoclast formation assay is performed at least 3 times using cells isolated independently from different cohorts of mice.

METHOD EXAMPLE. Osteoclast migration assay. Migration of osteoclasts is evaluated using a transwell assay as described previously with minor modifications. TRACP-positive cells are isolated from BMMNCs, which were previously cultured in M-CSF and RANKL for 6 days. The cells are trypsinized using 0.05% trypsin and 0.2% EDTA in Hank's balanced salt solution (HBSS). Equivalent numbers of TRACP-positive cells are loaded onto the upper chamber of transwells and allowed to migrate to the bottom chamber through an 8-μm polycarbonate filter coated with vitronectin (Takara Bio Inc., Otsu, Shiga, Japan). The bottom chamber contains α-MEM containing 1% bovine serum albumin (BSA) and M-CSF (30 ng/ml), and the reaction takes place for 6 h in a humidified incubator at 37°C. The number of TRACP-positive cells in the lower chamber is then counted using a Nikon TE 2000-S microscope (Nikon Instruments Inc., Melville, NY) (FIG. 7A).

METHOD EXAMPLE. Osteoclast adhesion assay. Osteoclast precursors (1×10^5 cells/mL) are placed into 96-well plates that are coated with 20 μg/mL vitronectin supplemented with M-CSF (30 ng/mL), as described previously. After 30 min of incubation, the wells are washed with phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde at room temperature for 10 to 15 min. Nonattached cells are gently removed with PBS, and adherent cells to $\alpha_v\beta_3$ are fixed with crystal violet. The number of adherent cells is counted under the phase contract microscope (FIG. 7B).

METHOD EXAMPLE. Tissue collection. Animals are sacrificed after 8 weeks. The femurs, tibias and lumbar spines are harvested and fixed in 10% neutral buffered formalin. After 48 h, the fixed samples are transferred to 70% alcohol for storage.

METHOD EXAMPLE. Colonogenic progenitor assay. Colony-forming unit-macrophage/monocyte (CFU-M) of BMMNCs is assayed as described previously. BMMNCs are isolated from mice by flushing the iliac bone marrow followed with Ficoll density gradient centrifugation. Approximately 2.5×10^4 BMMNCs are seeded onto a 35-mm gridded dish, which is composed of methylcellulose supplemented with 30 ng/mL M-CSF, and 20 ng/mL RANKL. Cells are cultured at 37°C in a 5% CO_2 incubator for 7 days. Compounds described herein are evaluated as a dose response, such as salubrinal (1, 2, and 5 μM), as administered and the number of colonies is counted using an inverted light microscope (FIG. 8).

METHOD EXAMPLE. Osteoblast differentiation assay. To induce osteogenic differentiation, MSCs from three groups of mice (sham OVX, OVX, and compound-treated OVX mice) are plated at 2×10^6/mL in osteogenic differentiation medium (MesenCult proliferation kit supplemented with 10 nM dexamethasone, 50 μg/mL ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate) in 6-well plates. Cells are maintained in osteogenic differentiation medium for 2 weeks, and the medium is changed every other day. For ALP staining, cells are fixed in citrate-buffered acetone for 30 s, incubated in alkaline-dye mix for 30 min, and counterstained with Mayer's Hematoxylin for 10 min. Cells are then evaluated microscopically, and the intensity of ALP staining is determined (FIG. 9).

METHOD EXAMPLE. (CFU-F) Assay. To measure the frequency of MSCs in bone marrow cell, a CFU-F assay is performed as previously reported with minor modification. Briefly, BMMNCs are separated by Ficoll-Hypaque density gradient centrifugation. Approximately 2×10^6/mL BMMNCs are plated onto 6-well tissue culture plates in triplicate in 2 mL of complete MesenCult medium, and incubated at 37°C. At day 14 of culture, medium is removed followed by two washes of PBS and staining with a HEMA-3 quick staining kit (Fisher Scientific) according to the manufacturer's instructions. The number of colonies with more than 50 cells is counted microscopically at 20× magnification using a phase contrast

microscope (Nikon, Fryer INC. Chicago, IL). Colonies, which do not present MSC-like morphology are excluded (FIG. 10).

METHOD EXAMPLE. X-ray imaging and histology: For analysis of microstructure of bone as well as determination of BMD and BMC, μCT imaging using a desktop μCT-20 (Scanco Medical) and pQCT imaging (XCT Research SA) is conducted (Zhang and Yokota 2007). Total bone mineral density (BMD_t; mg/cm^3) and cortical BMD (BMD_c; mg/cm^3) is computed together with total bone area (A_t; mm^2) and cortical area (A_c; mm^2) (Zhang *et al*. 2007a; Zhang and Yokota 2011).

METHOD EXAMPLE. Histology: New bone formation is evaluated using calcein (fluorochrome dye) and bone resorption is evaluated using using TRCAP staining. Mice are given an intraperitoneal injection of calcein at 30 μg/g body mass on 1 and 2 weeks prior to harvest. The transverse sections (~ 80 μm in thickness) are removed from the femoral shaft and lumber spine using a diamond-embedded wire saw. After polishing the surface bone sections are mounted on standard microscope slides. The mineralizing surface, mineral apposition rate, and bone formation rate are determined (Zhang *et al*. 2006a&b, 2007b). For histomorphometric analysis, the femurs are fixed in 4% paraformaldehyde for 2 days and then decalcified in 20% EDTA for 2 weeks. Decalcified bones are embedded in paraffin, and 5-mm-thick sagittal sections are cut (Zhang *et al*. 2010). To quantify osteoclasts *in vivo*, tartrate-resistant acid phosphatase (TRACP) staining is performed (Leung *et al*. 2010; Uveges *et al*. 2008). For quantification of the cancellous surfaces occupied by osteoclasts, the lengths of bone surfaces occupied by $TRACP^+$ multinucleated osteoclasts is determined, and normalized to bone surface (BS) within the region of interest. Quantitative analysis of trabecular bone at the distal femur is performed using coronal sections with haematoxilin Eosin (H&E) staining (Pierroz *et a*l. 2012; Kamiya *et al*. 2008). Morphometric parameters are used for evaluation BV/TV (in %, TV, total tissue area, calculated from the total tissue area, and BV, trabecular bone area, calculated from the total trabecular area) using a Metamorph image analysis system.

METHOD EXAMPLE. Statistical analysis. Data are expressed as mean ± standard error of mean (SEM). Statistical significance among the experimental groups is examined using one-way ANOVA. For pair-wise comparisons a post-hoc test is conducted using Fisher's protected least significant difference. A paired t-test is employed to evaluate statistical significance between the compound treated samples, such as salubrinal treated samples, and non-treated control samples. All comparisons are two-tailed and statistical significance is assumed at $p < 0.05$. The asterisks (*, **, and ***) represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

METHOD EXAMPLE. Attenuation of OVX symptoms in the salubrinal treated

OVX mice. Compared to the sham OVX mice, the OVX mice gained body weight by 13% ($p < 0.001$) (FIG. 1A), and administration of salubrinal suppressed the increase in body weight ($p < 0.001$) (FIG. 1B).

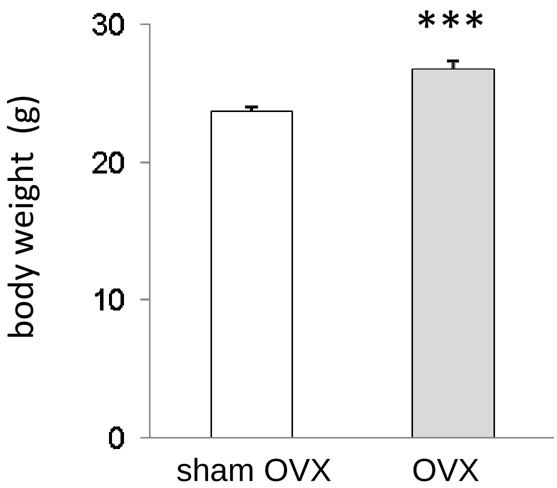


FIG. 1A

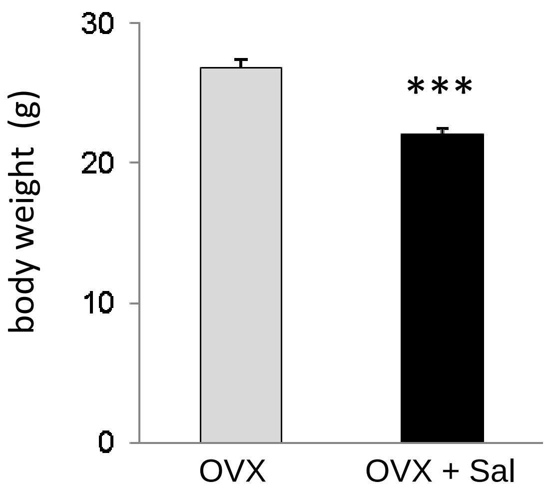


FIG. 1B

In the OVX mice uterine weight was 76% ($p < 0.001$) lower than the sham OVX mice (FIG. 2A). However, administration of salubrinal reduced the OVX-driven decrease in uterus weight to 44% ($p < 0.001$) (FIG. 2B).

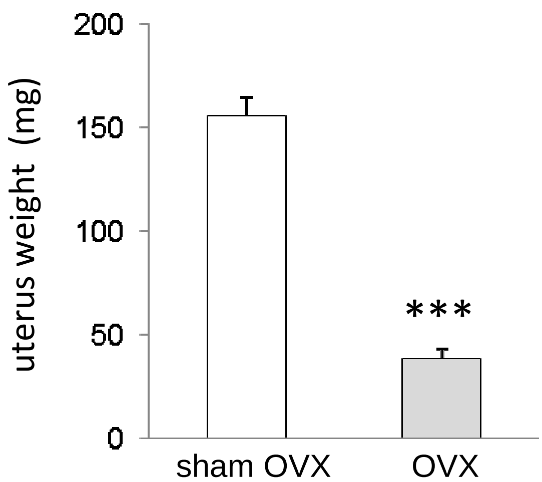


FIG. 2A

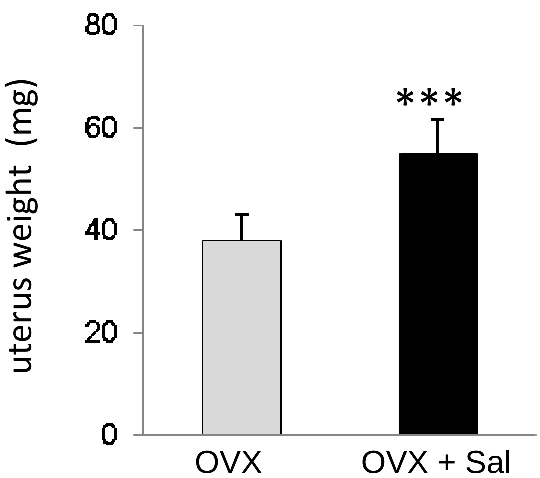


FIG. 2B

METHOD EXAMPLE. Suppression of fat in the salubrinal treated OVX mice. During 8 weeks after ovariectomy, the amount of total fat was increased from 20.32 ± 1.96% in the sham OVX mice to 27.61 ± 1.27% in the OVX mice ($p < 0.01$) (FIG. 3A). Consistent with the amount of total fat, an increase in abdominal fat was 22.98 ± 3.11% in the sham OVX mice and 36.21 ± 1.75% in the OVX mice ($p < 0.01$) (FIG. 3B).

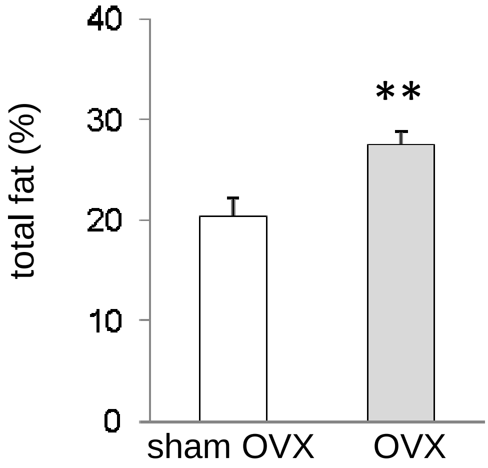


FIG. 3A

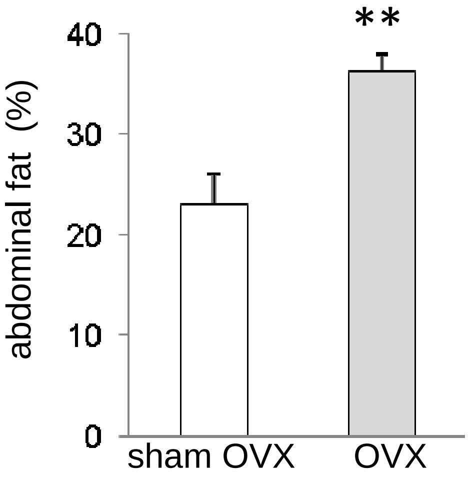


FIG. 3B

During 4 weeks of salubrinal treatment, total fat was reduced by 13% from 27.61 ± 1.27% (OVX mice) to 24.03 ± 0.95% (salubrinal treated OVX mice) ($p < 0.05$) (FIG. 3C), abdominal fat from 36.21 ± 1.75% (OVX mice) to 27.36 ± 1.07% (salubrinal treated OVX mice) ($p < 0.001$) (FIG. 3D).

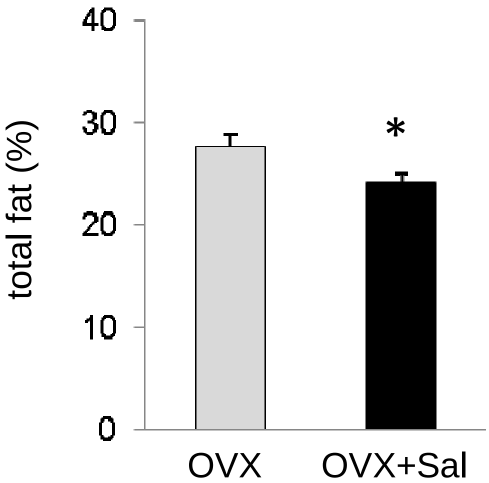


FIG. 3C

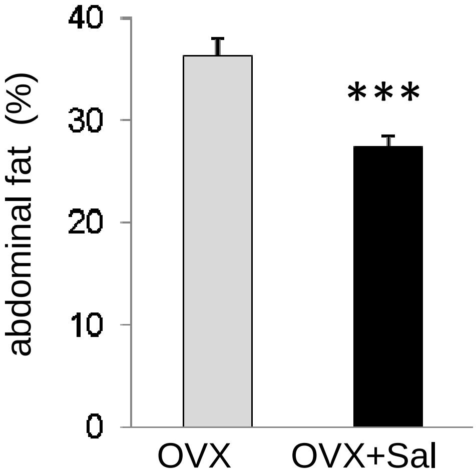


FIG. 3D

METHOD EXAMPLE. Reduction in BMD/BMC in the OVX mice. In the period of 8 weeks, ovariectomy decreased BMD by 10.8% (whole body), 11.7% (lumbar spine), 2.7% (femur), and 3.3% (tibia) (FIG. 4A). In the OVX mice, BMC was also decreased by 12.7% (whole body), 10.0% (lumbar spine), 11.0% (femur), and 4.6% (tibia) (FIG. 4B).

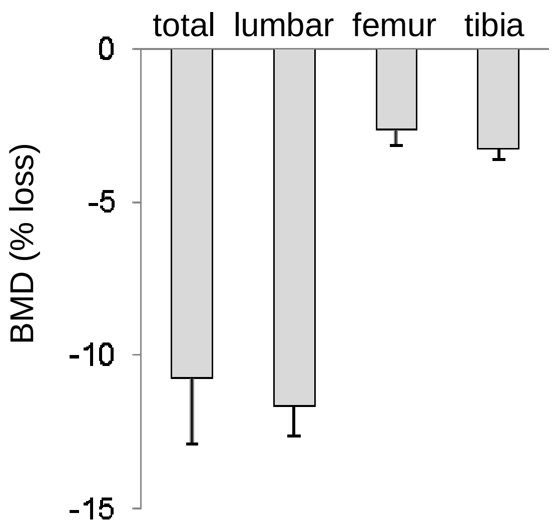


FIG. 4A

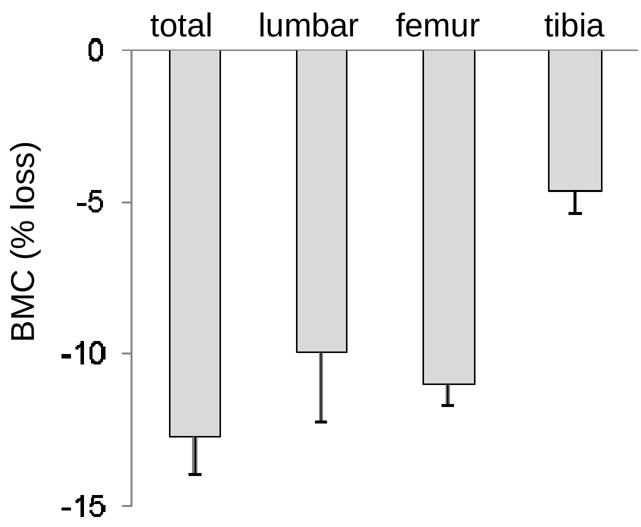


FIG. 4B

The observed reduction in BMD and BMC in the OVX mice supported effectiveness of ovariectomy surgery in this study.

METHOD EXAMPLE. Increase in BMD and BMC in the salubrinal treated OVX mice. Compared to the OVX mice, salubrinal treated OVX mice elevated BMD in the lumbar spine from 0.0406 ± 0.0011 g/cm^2 (OVX mice) to 0.0432 ± 0.0007 g/cm^2 (salubrinal treated OVX) ($p < 0.05$) (FIG. 4C). Likewise, salubrinal treated OVX mice presented a significant increase in BMC in the lumbar spine from 0.0466 ± 0.0022 g (OVX mice) to 0.0597 ± 0.0035 g (salubrinal treated OVX) ($p < 0.001$) (FIG. 4D).

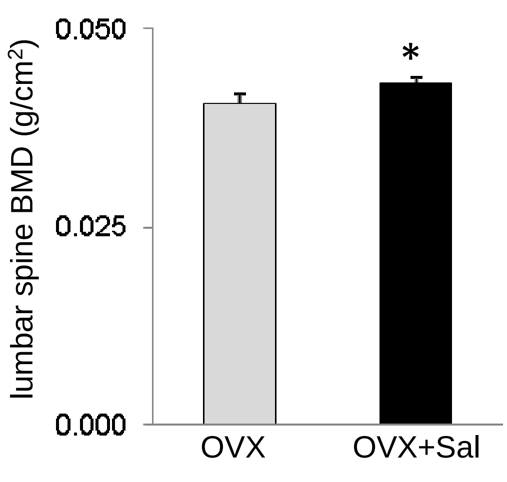


FIG. 4C

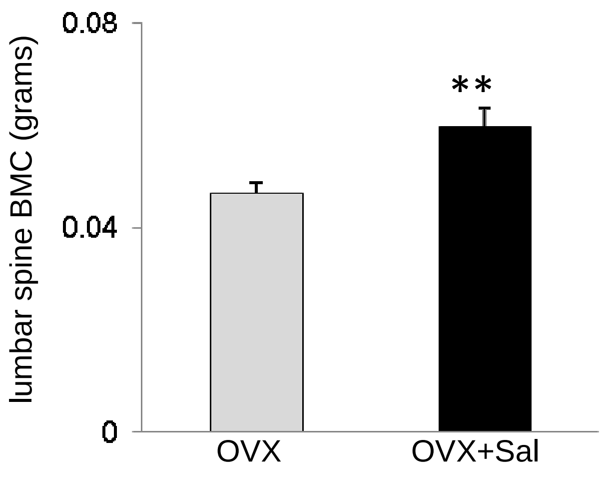


FIG. 4D

METHOD EXAMPLE. Suppression of osteoclast development by administration of salubrinal. Compared to the sham OVX mice, the OVX mice exhibited an increase in the area of osteoclasts and the salubrinal treated OVX mice presented substantial reduction in this area (FIG. 5).

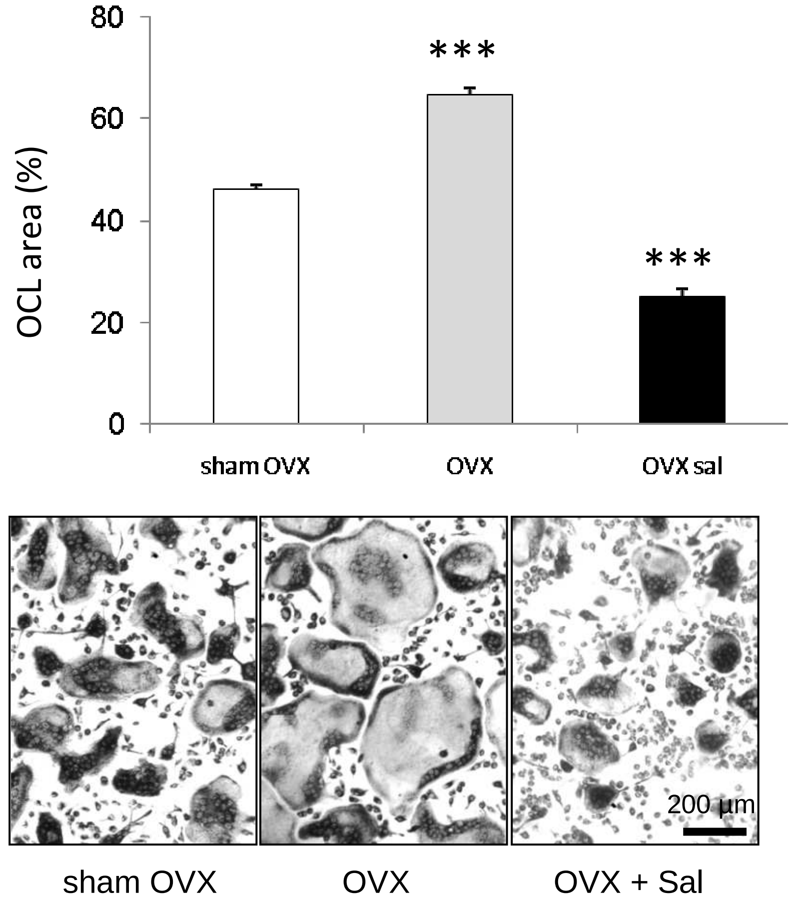


FIG. 5

The osteoclast area was 46.11 ± 1.02% (sham OVX mice), 64.56 ± 1.56% (OVX mice), and 24.94 ± 1.78% (salubrinal treated OVX mice).

METHOD EXAMPLE. Salubrinal's dosage dependent effects on osteoclast development. To evaluate the role of salubrinal in osteoclast differentiation with BMMNCs harvested from the sham OVX mice, three dosages of salubrinal (1, 2, and 5µM) were employed. Throughout 6 days of culture, salubrinal was present in media. Compared to the vehicle treated cultures, administration of salubrinal resulted in a significant decrease in osteoclast differentiation as evidenced by reduced percent of osteoclast area (FIG. 6A, left bars, ***$p < 0.001$ for 1, 2, and 5µM of salubrinal).

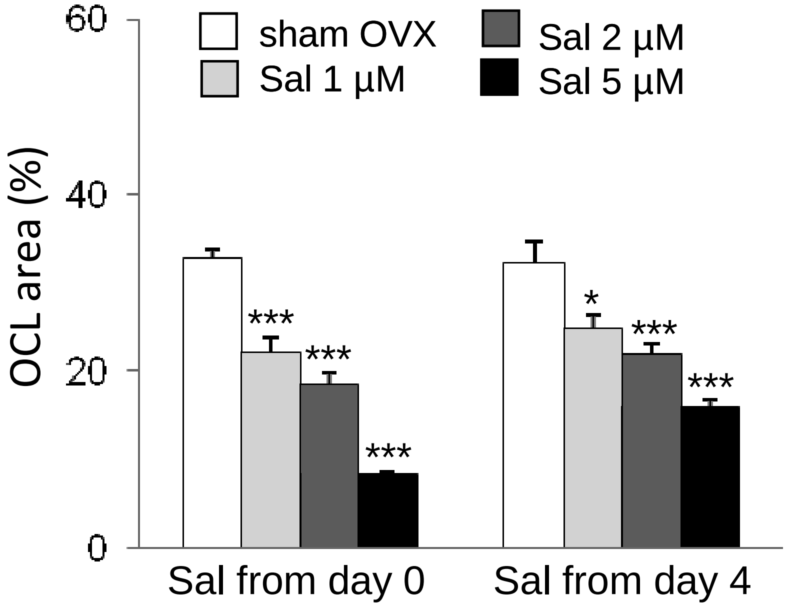


FIG. 6A

To test the effects of salubrinal on osteoclast maturation, salubrinal was administered on day 4 with M-CSF and RANKL. As shown in FIG. 6A, right bars, a significant decrease in percent osteoclast area was observed (*$p < 0.05$ for 1 µM, and ***$p < 0.001$ for 2 µM and 5µM of salubrinal vs. vehicle controls). A dosage dependent response was observed in these experiments.

METHOD EXAMPLE. Using BMMNCs harvested from the OVX mice, the role of salubrinal on osteoclast differentiation was assessed with three dosages of salubrinal (1, 2, and 5µM). Compared to the vehicle treated osteoclast cultures, administration of salubrinal resulted in a significant decrease in osteoclast differentiation as evidenced by reduced percent of osteoclast area (FIG. 6B, left bars, ***$p < 0.001$ for 1, 2 and 5µM of salubrinal).

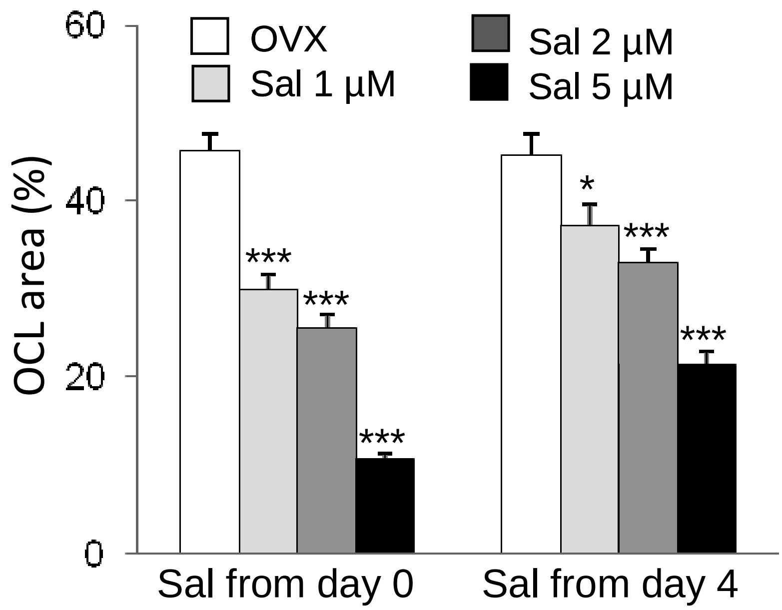


FIG. 6B

To test the effects of salubrinal on osteoclast maturation of BMMNCs from the OVX mice, salubrinal was administered on day 4 with M-CSF and RANKL. As shown in FIG. 6B, right bars, a significant decrease in percent osteoclast area was observed with all dosages (*$p < 0.05$ for 1μM, and ***$p < 0.001$ for 2 and 5μM of salubrinal vs. vehicle controls). As shown in the images in Fig. 6, the OVX samples showed more active in all stages of osteoclast development than the sham OVX samples and the salubrinal treated samples inactivated its development in a dosage dependent manner.

METHOD EXAMPLE. Salubrinal driven suppression of migration and adhesion of osteoclasts. Preosteoclast cells isolated from the OVX mice were more active in migration (86.0 ± 5.6 cells) than those from the sham OVX mice (38.7 ± 2.0 cells) ($p < 0.001$). Compared to the preosteoclast cells isolated from the OVX control, administration of salubrinal reduced migration (56.6 ± 4.1 cells) ($p < 0.001$) (FIG. 7A).

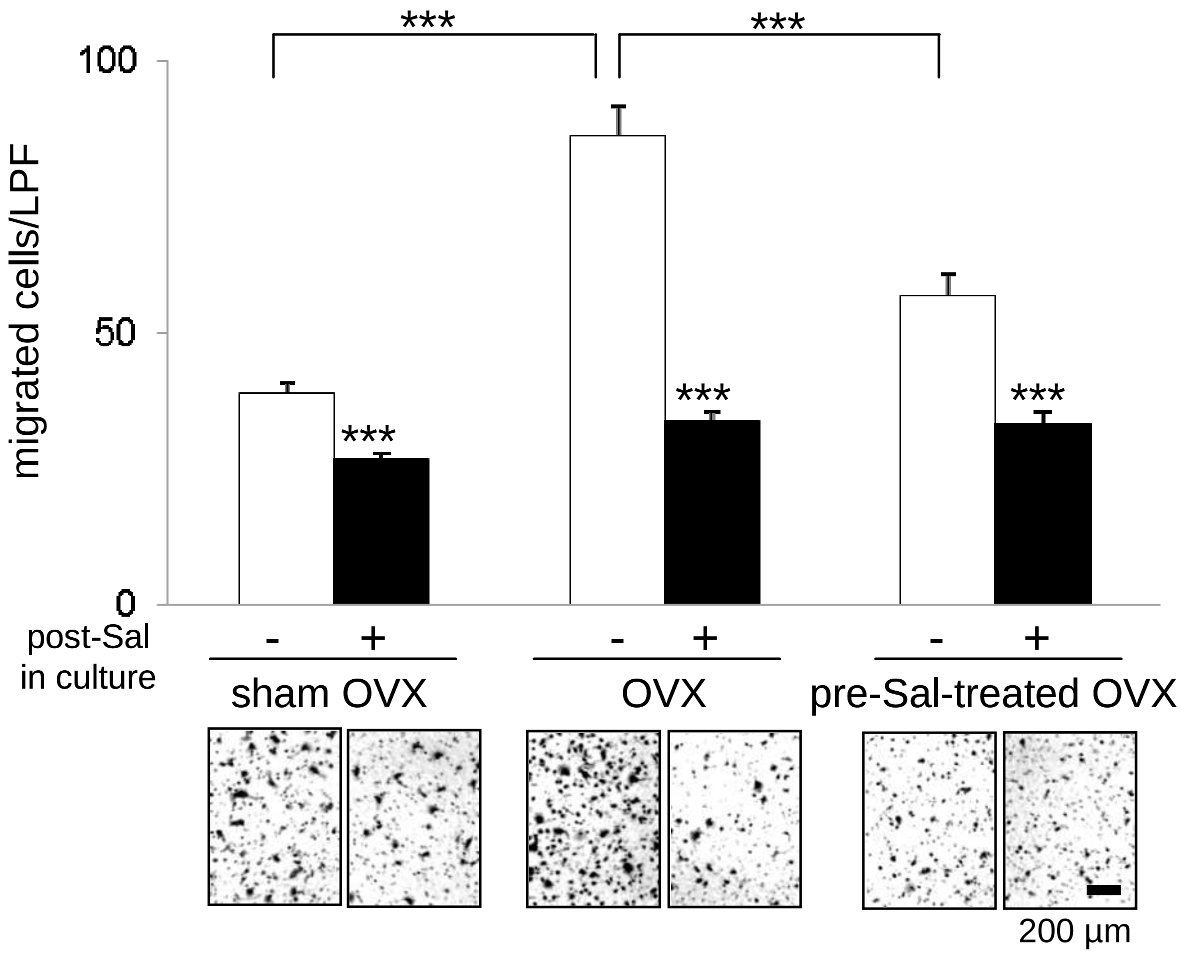


FIG. 7A

Quantitative evaluation of migration in response to M-CSF was also conducted in the presence and absence of administration of salubrinal in culture using cells isolated from three groups of mice (sham OVX, OVX, and salubrinal treated OVX). Compared to the cultures in the absence

of salubrinal, salubrinal-treated preosteoclast cells migrated at a lower pace as shown by representative microphotographs of the migrated cells and by quantification (all ***p < 0.001 in these three groups of mice).

METHOD EXAMPLE. Reduction in osteoclast adhesion by salubrinal. In M-CSF mediated adhesion of osteoclasts to $\alpha_V\beta_3$, the OVX mice presented greater adhesion (72.1 ± 4.5 cells) that the sham OVX mice (62.0 ± 2.5 cells) (p < 0.05) (FIG. 7B).

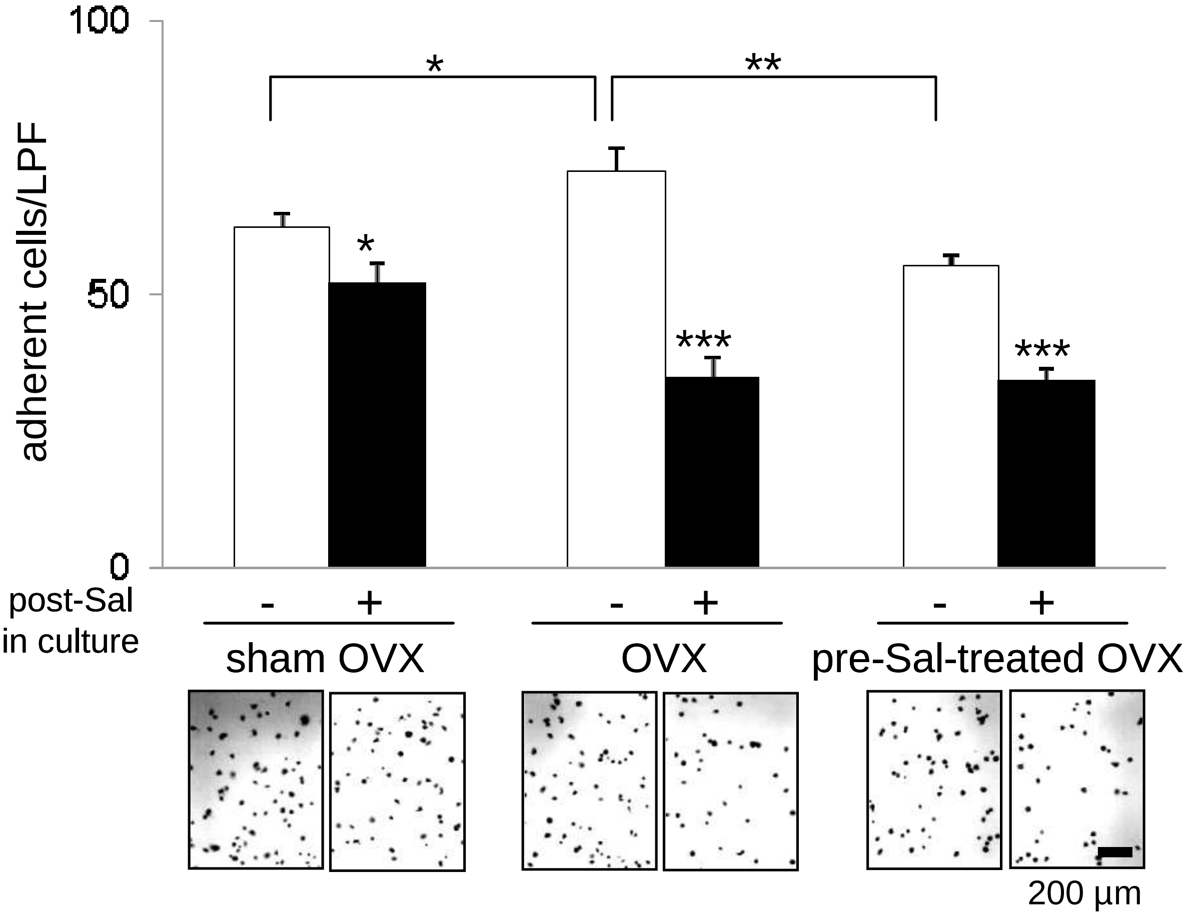


FIG. 7B

Compared to the OVX mice, salubrinal administration for 4 weeks in the salubrinal treated OVX mice reduced osteoclast adhesion (55.3 ± 1.8 cells) (p < 0.01). In addition, post administration of salubrinal in the culture medium reduced M-CSF-mediated adhesion in cells isolated from the sham OVX mice (p < 0.05), OVX mice (p < 0.001), and salubrinal treated OVX mice (p < 0.001).

METHOD EXAMPLE. Reduction in the population of osteoclast progenitors by salubrinal. To determine the effects of salubrinal on the population of osteoclast progenitors, a colony-forming unit-macrophage assay was performed using BMMNCs isolated from the sham OVX mice. Compared to vehicle control, salubrinal at 1, 2, and 5 µM reduced the total number of CFU-M in the iliac (***p < 0.001 in three dosages) in a dosage-dependent manner (FIG.

8A).

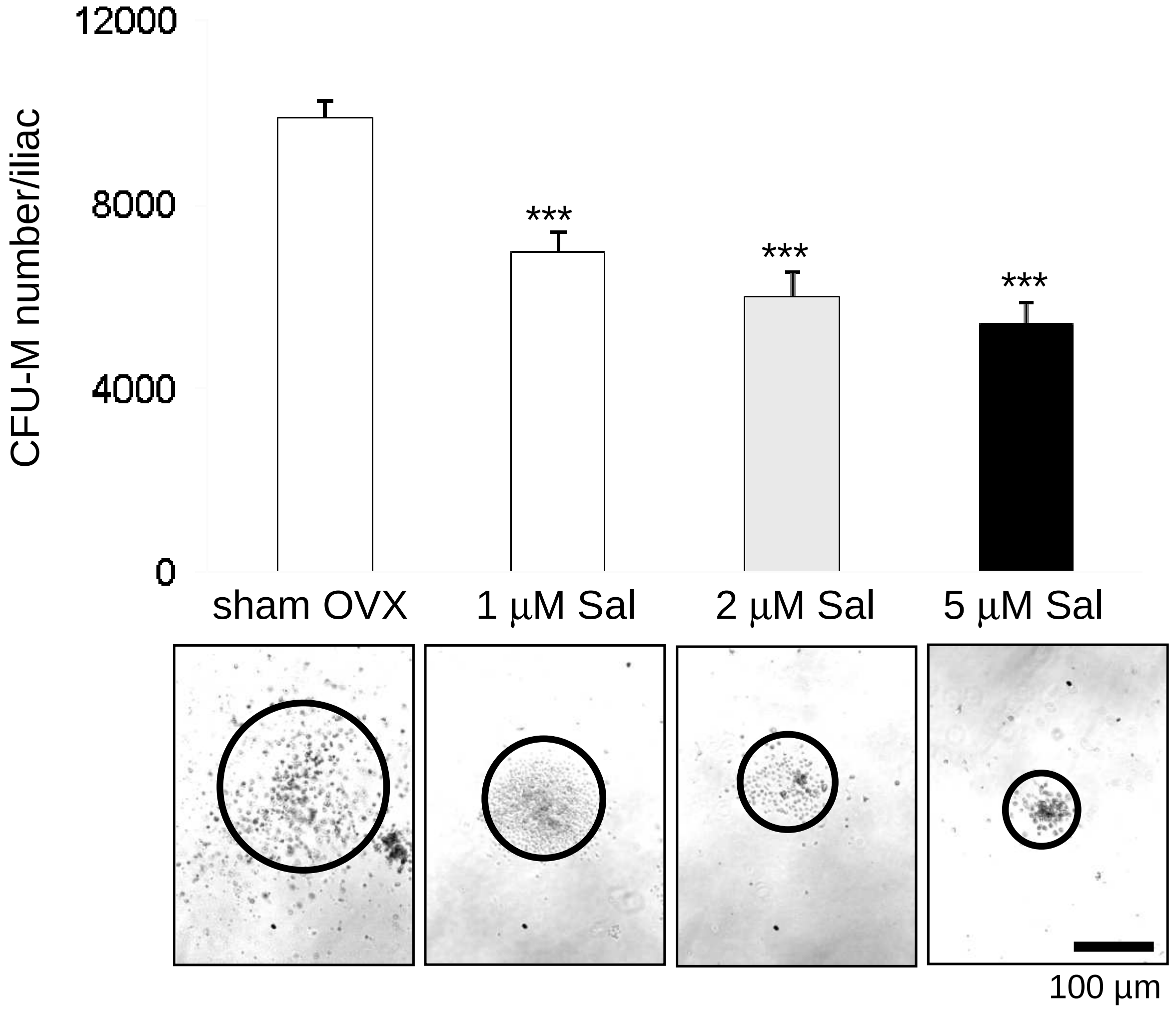


FIG. 8A

To assess the effect of salubrinal on the frequency of CFU-M, the comparison of CFU-M numbers among three groups of mice (sham OVX, OVX, and salubrinal treated OVX mice) was conducted (FIG. 8B).

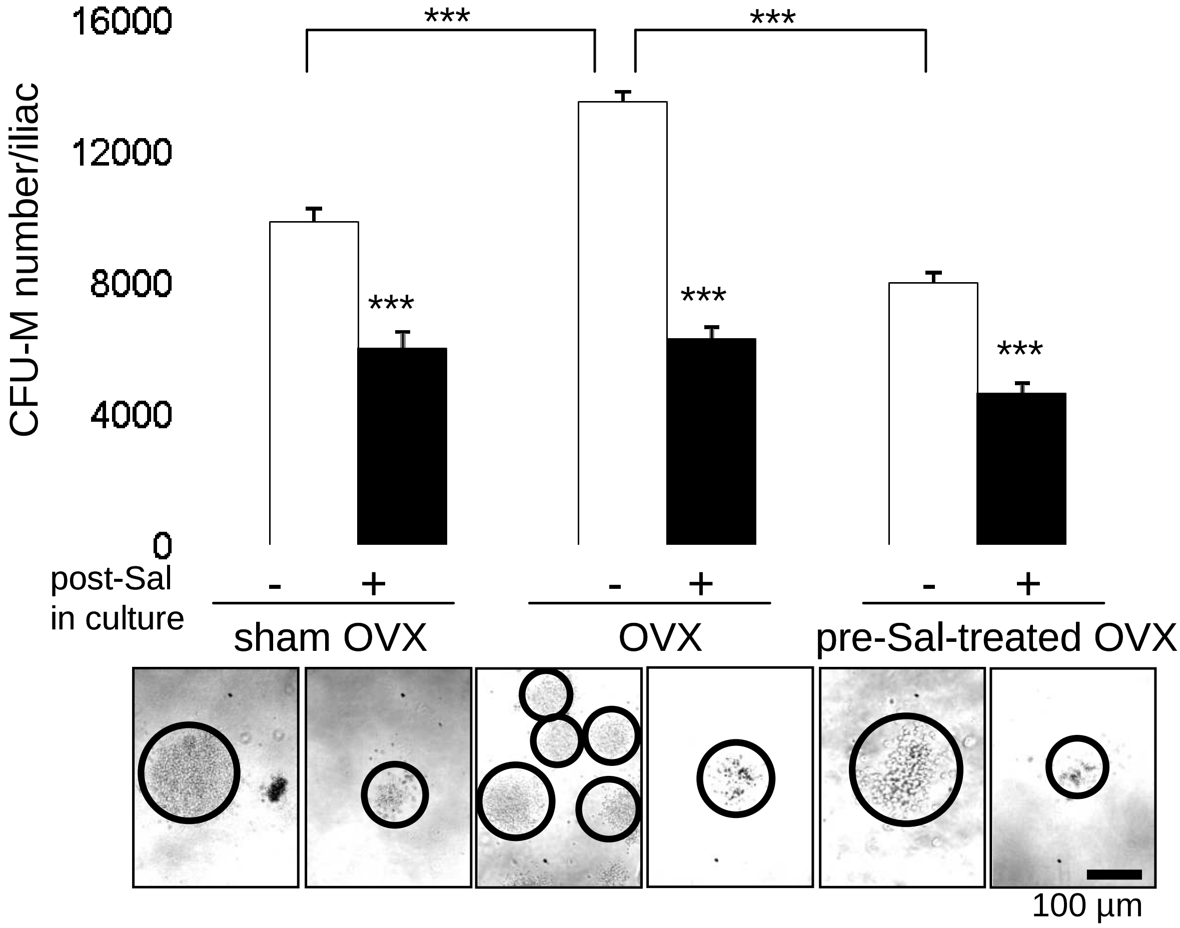


FIG. 8B

The result showed that the cells isolated from the OVX mice was more stimulated in the frequency of CFU-M (13526 ± 330 number/iliac) than those from the sham OVX mice (9892 ± 383 number/iliac) ($p < 0.001$), and 4-week salubrinal treatment reduced the number (8041 ± 282 number/iliac) compared to the OVX mice ($p < 0.001$). Furthermore, the post salubrinal (2 µM) treatment in culture for 7 days reduced the numbers of colonies in all three groups ($p < 0.001$).

METHOD EXAMPLE. Promotion of osteoblast differentiation by salubrinal. The effect of salubrinal on differentiation of osteoblasts was examined with a colony-forming unit-osteoblast (CFU-OBL) assay. MSCs were isolated from 3 groups of mice (sham OVX, OVX, and salubrinal treated OVX), and ALP staining was used to determine ALP activity. The result shows that a significant increase in ALP positive cells in CFU-OBL was detected in the salubrinal treated OVX mice (66.9 ± 4.9%), compared to the sham OVX mice (34.5 ± 5.3%) and the OVX mice (33.9 ± 4.9%) (both $p < 0.001$) (FIG. 9A).

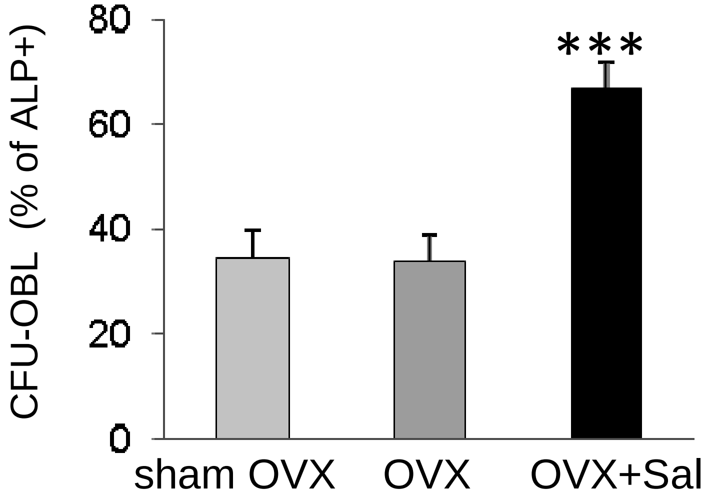


FIG. 9A

In addition, the effect of post-administration of salubrinal (0.5 μM) was evaluated for MSCs isolated 2, 4, and 4 weeks after ovariectomy (FIG. 9B).

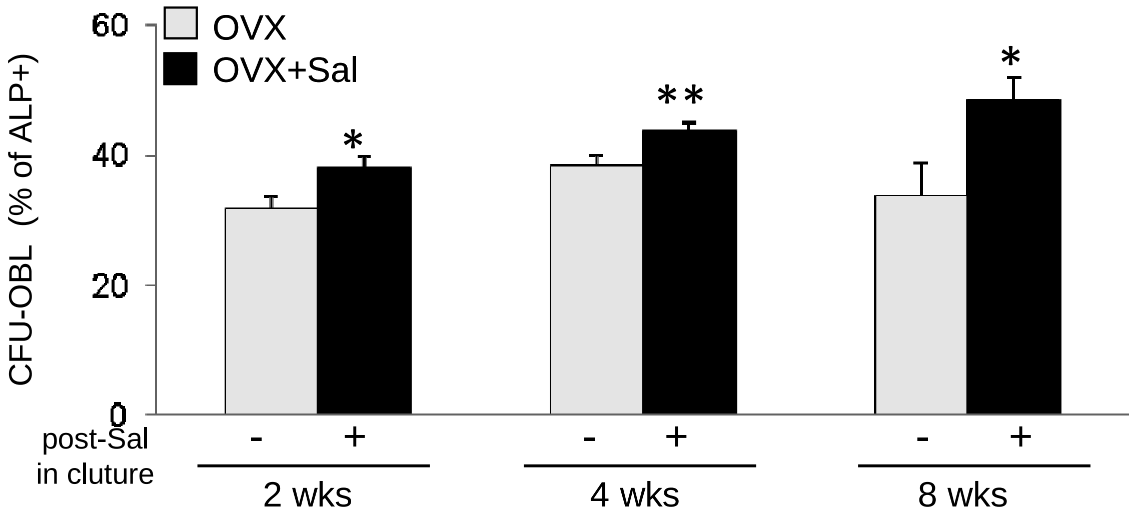


FIG. 9B

In this time-course experiment, post-salubrinal administration enhanced CFU-OBL (percent of ALP positive cells) in all cases (p < 0.05 in 2 weeks, p < 0.01 in 4 weeks, and p < 0.05 in 8 weeks).

METHOD EXAMPLE. Increase in colony forming unit-fibroblast by salubrinal. In the CFU-F assay, BMMNCs from three groups of mice (sham OVX, OVX, and salubrinal treated OVX) were cultured for 2 weeks and the number of CFU-F colonies were counted. The result showed that per 2 X 10^6 bone marrow cells the salubrinal treated OVX mice presented a significant increase in the colony numbers (41.6 ± 2.2), compared to the sham OVX mice (25.4 ± 3.0; p < 0.001) and the OVX mice (29.8 ± 2.8; p < 0.01) (FIG. 10A).

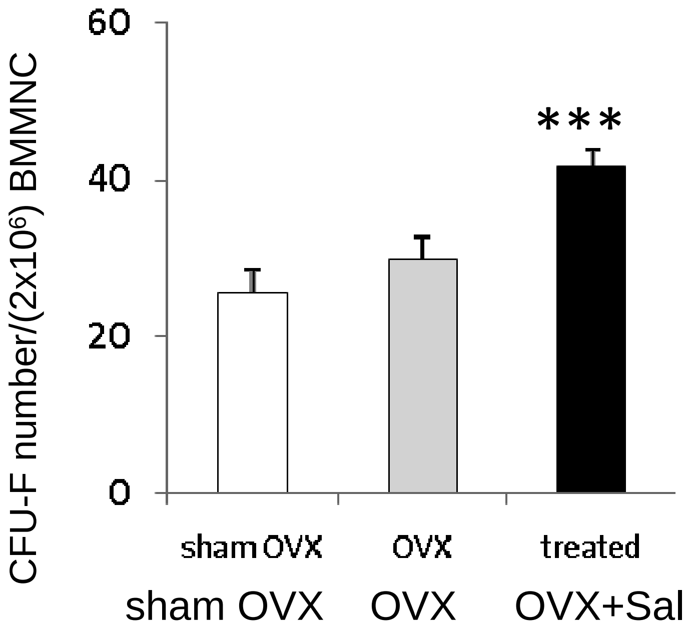


FIG. 10A

Furthermore, the effect of post-administration of salubrinal (0.5 μM) elevated the colony number in all three groups, and a significance increase was detected in the OVX mice ($p < 0.05$) (FIG. 10B).

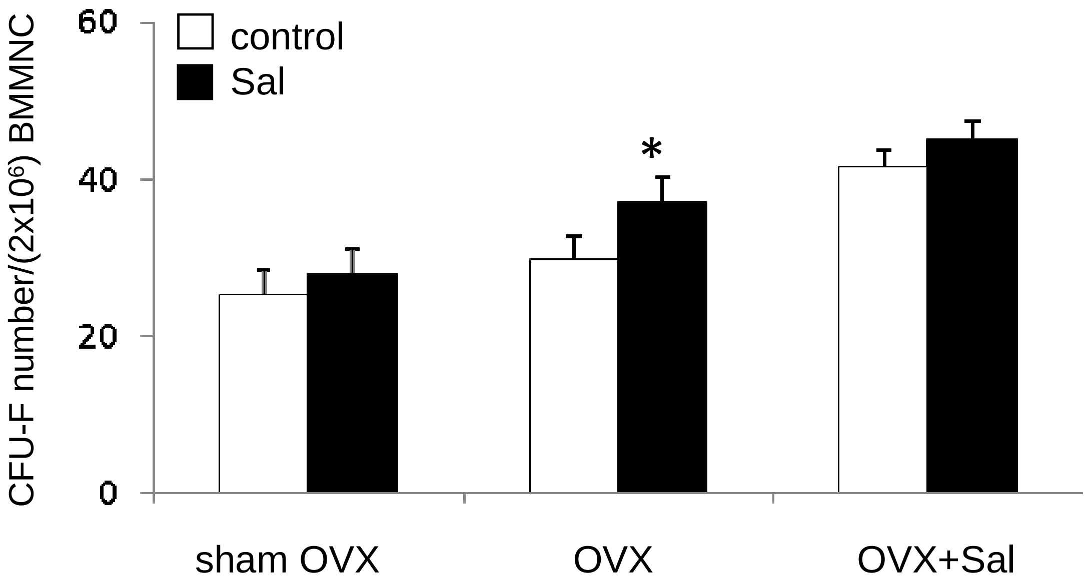


FIG. 10B

METHOD EXAMPLE. Effects of salubrinal on BMD of hindlimb-suspended mice. The assay is described in Sugiyama, *et al*. 2006 and Greising *et al*. 2011, the disclosures of which are incorporated herein by reference. Hindlimb-suspended mice (female, ~ 6 weeks old) show reduce BMD in the femur and lumbar spine (approximately 10% reduction) (Saxena *et al*. 2011) (FIG. 12).

METHOD EXAMPLE. H&E staining of the distal femur**.** Total tissue area

(TV) and trabecular bone area (BV) are measured. Reductions of trabecular bone volume fraction BV/TV (%) are observed by unloading (FIG. 12A).

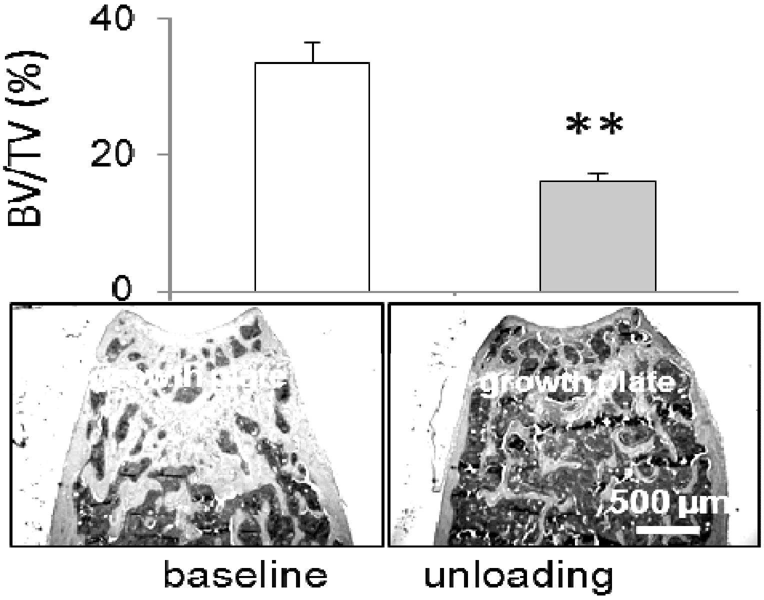


FIG. 12A

METHOD EXAMPLE. Effects of salubrinal on BMD of the femur and lumber spine of the unloaded mice (% change). The reduction of trabecular bone volume fraction BV/TV (%) by unloading, and the corresponding negative change in BMD is suppressed by administration of compounds described herein, including salubrinal (1 mg/kg) for 2 weeks (FIG. 12B).

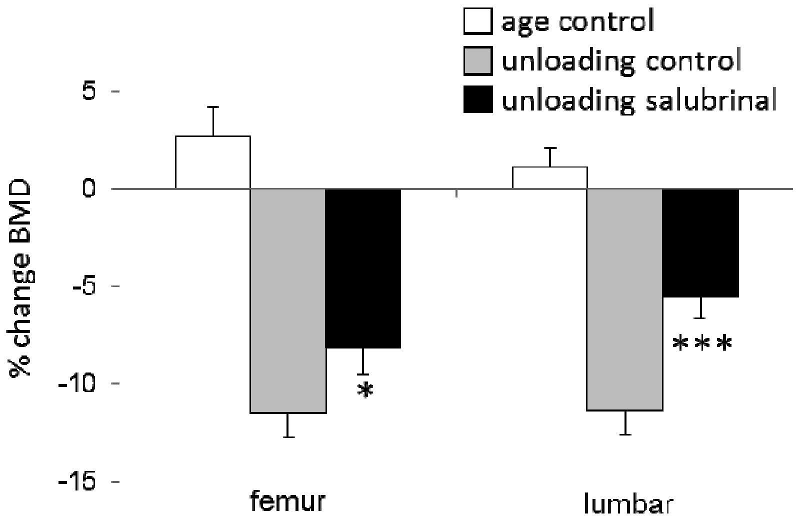


FIG. 12B

The following publications, and each of the additional publications cited herein are incorporated herein by reference:

1. Zhang P, Yokota H (2011) Knee loading stimulates healing of mouse bone wounds in a femur neck. Bone 49:867-872.

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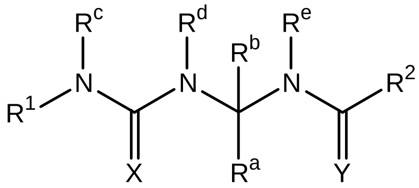
WHAT IS CLAIMED IS:

1. A composition for treating a metabolic disorder in a host animal, the composition comprising one or more compounds of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

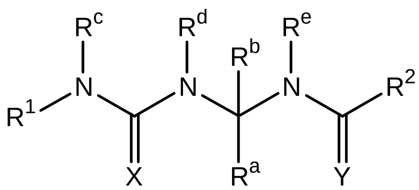
R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

2. A method for treating a metabolic disorder in a host animal, the method comprising the step of administering to the host animal a therapeutically effective amount of one or more compounds of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

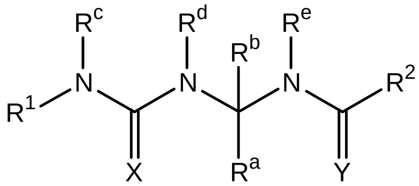
R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo;

or a composition thereof further comprising one or more carriers, diluents, or excipients, or a combination thereof.

3. Use of a therapeutically effective amount of one or more compounds in the manufacture of a medicament for treating a metabolic disorder in a host animal, where the one or more compounds are of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

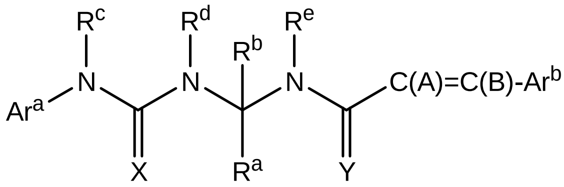
4. The composition or use or method of any one of claims 1 to 3 wherein R^1 is alkyl, aryl, or heteroaryl, each of which is optionally substituted.

5. The composition or use or method of any one of claims 1 to 3 wherein R^1 is aryl or heteroaryl, each of which is optionally substituted.

6. The composition or use or method of any one of claims 1 to 5 wherein R^2 is alkenyl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

7. The composition or use or method of any one of claims 1 to 5 wherein R^2 is alkenyl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

8. The composition or use or method of any one of claims 1 to 3 wherein the inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X and Y are independently O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1-C_6 alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, acyl, and a prodrug capable of releasing the attached nitrogen in vivo to form the corresponding H or salt derivative thereof;

and A and B are independently H, or optionally substituted C_1-C_6 alkyl

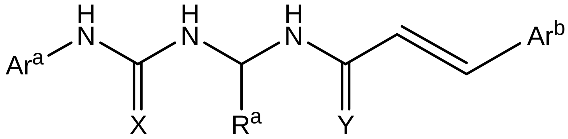
9. The composition or use or method of claim 8 wherein the aryl is a bicyclic aryl.

10. The composition or use or method of claim 8 or 9 wherein the heteroaryl is a bicyclic heteroaryl.

11. The composition or use or method of any one of claims 11 to 13 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 haloalkyl, C_1-C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

12. The composition or use or method of any one of claims 8 to 11 wherein the alkenyl has an E geometry.

13. The composition or use or method of any one of claims 1 to 3 wherein the inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein,

X is O or S;

Y is O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted; and

R^a is optionally substituted alkyl.

14. The composition or use or method of claim 13 wherein the aryl is a bicyclic aryl.

15. The composition or use or method of claim 13 or 14 wherein Ar^a is a bicyclic heteroaryl.

16. The composition or use or method of any one of claims 13 to 15 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 haloalkyl, C_1-C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

17. The composition or use or method of any one of claims 13 to 16 wherein R^a is haloalkyl.

18. The composition or use or method of any one of claims 13 to 17 wherein R^a is not trifluoromethyl.

19. The composition or use or method of any one of claims 13 to 18 wherein R^a is haloalkyl, where halo is selected from the group consisting of chloro and bromo, and combinations thereof.

20. The composition or use or method of any one of claims 13 to 19 wherein R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C_1-C_6 alkyl, and acyl.

21. The composition or use or method of any one of claims 1 to 3 wherein the inhibitor is salubrinal, or an analog or a derivative thereof, or a pharmaceutically acceptable salt of the foregoing.

22. The composition or use or method of any one of claims 1 to 21 wherein the medicament further comprises one or more carriers, diluents, or excipients, or a combination thereof.

23. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is osteoporosis.

24. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is osteopenia.

25. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is bone remodeling dysfunction.

26. The composition or use or method of any one of the preceding claims wherein the composition is capable of increasing BMD, BMC, or a combination thereof in the host animal.

27. The composition or use or method of any one of the preceding claims wherein the composition is capable of increasing BMD, BMC, or a combination thereof in the lumbar spine host animal.

28. The composition or use or method of any one of the preceding claims wherein the composition is capable of increasing osteoblast differentiation.

29. The composition or use or method of any one of the preceding claims wherein the composition is capable of increasing osteoblast function.

30. The composition or use or method of any one of the preceding claims wherein the composition is capable of decreasing osteoclast differentiation.

31. The composition or use or method of any one of the preceding claims wherein the composition is capable of decreasing osteoclast function.

32. The composition or use or method of any one of the preceding claims wherein the composition is capable of increasing CFU-OBL in the host animal.

33. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is a fat metabolism dysfunction.

34. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is a fat storage dysfunction.

35. The composition or use or method of any one of the preceding claims wherein the metabolic disorder is obesity.

36. The composition or use or method of any one of the preceding claims wherein the composition is capable of maintaining or decreasing fat load in the host animal.

37. The composition or use or method of any one of the preceding claims wherein the composition is capable of maintaining or decreasing abdominal fat load in the host animal.

38. The composition or use or method of any one of the preceding claims wherein the composition is capable of decreasing adipocyte differentiation.

39. The composition or use or method of any one of the preceding claims wherein the composition is capable of decreasing adipocyte cell division.

40. The composition or use or method of any one of the preceding claims wherein the metabolic disorder results in dysuria

41. The composition or use or method of claim 40 wherein the dysuria is urinary urgency.

42. The composition or use or method of claim 40 or 41 wherein the dysuria is urinary frequency.

43. The composition or use or method of any one of the preceding claims wherein the host animal is female.

44. The composition or use or method of claim 43 wherein the metabolic disorder is post-menopausal osteoporosis.

45. The composition or use or method of claim 43 or 44 wherein the metabolic disorder is post-menopausal osteopenia.

46. The composition or use or method of any one of claims 43 to 45 wherein the metabolic disorder is post-menopausal obesity.

47. The composition or use or method of any one of claims 43 to 46 wherein the composition is capable of maintaining or increasing uterine weight load.

48. The composition or use or method of any one of claims 1 to 47 wherein the host animal is a human.

BMC Musculoskeletal Disorders

RESEARCH ARTICLE Open Access

Effects of salubrinal on development of osteoclasts and osteoblasts from bone marrow-derived cells

Hiroki Yokota[1,2*], Kazunori Hamamura[1], Andy Chen[1], Todd R Dodge[1], Nancy Tanjung[1], Aysan Abedinpoor[1] and Ping Zhang[1,2,3*]

Abstract

Background: Osteoporosis is a skeletal disease leading to an increased risk of bone fracture. Using a mouse osteoporosis model induced by administration of a receptor activator of nuclear factor kappa-B ligand (RANKL), salubrinal was recently reported as a potential therapeutic agent. To evaluate the role of salubrinal in cellular fates as well as migratory and adhesive functions of osteoclast/osteoblast precursors, we examined the development of primary bone marrow-derived cells in the presence and absence of salubrinal. We addressed a question: are salubrinal's actions more potent to the cells isolated from the osteoporotic mice than those isolated from the control mice?

Methods: Using the RANKL-injected and control mice, bone marrow-derived cells were harvested. Osteoclastogenesis was induced by macrophage-colony stimulating factor and RANKL, while osteoblastogenesis was driven by dexamethasone, ascorbic acid, and β-glycerophosphate.

Results: The results revealed that salubrinal suppressed the numbers of colony forming-unit (CFU)-granulocyte/macrophages and CFU-macrophages, as well as formation of mature osteoclasts in a dosage-dependent manner. Salubrinal also suppressed migration and adhesion of pre-osteoclasts and increased the number of CFU-osteoblasts. Salubrinal was more effective in exerting its effects in the cells isolated from the RANKL-injected mice than the control. Consistent with cellular fates and functions, salubrinal reduced the expression of nuclear factor of activated T cells c1 (NFATc1) as well as tartrate-resistant acid phosphatase.

Conclusions: The results support the notion that salubrinal exhibits significant inhibition of osteoclastogenesis as well as stimulation of osteoblastogenesis in bone marrow-derived cells, and its efficacy is enhanced in the cells harvested from the osteoporotic bone samples.

Keywords: Osteoporosis, RANKL, Salubrinal, Osteoclasts, Osteoblasts

Background

Osteoporosis is a common skeletal disease of bone loss, which leads to an increased risk of bone fractures, morbidity, mortality, and an economic burden to society [1-3]. In many cases it is a physiological consequence of the aging process [3,4], and in postmenopausal women it is induced by a decrease in the production of estrogen, a hormone known to maintain the appropriate ratio of bone-forming osteoblasts to bone-resorbing osteoclasts [5]. During the past 20 years, many therapeutic drugs have been developed to prevent osteoporotic bone loss. Bisphosphonates are the most widely prescribed medications to treat postmenopausal osteoporosis, but they may be associated with an increased risk of osteonecrosis of the jawbone and atypical femur fracture [6]. Other treatments include administration of estrogen and estrogen analogs, as well as parathyroid hormone. However, increased risks of breast cancers and blood clots have been reported as side effects of these treatments [7-9]. The aim of this study is to evaluate a therapeutic role of a chemical agent, salubrinal, in potential treatment of osteoporosis.

Salubrinal is a small chemical agent (480 Da, $C_{21}H_{17}Cl_3N_4OS$) known to block de-phosphorylation of

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eukaryotic translation initiation factor 2 alpha (eIF2α) [10]. Salubrinal is also reported to attenuate molecular signaling mediated by nuclear factor kappa B (NFκB) [11]. The elevated phosphorylation level of eIF2α upregulates activating transcription factor 4 (ATF4), one of the key transcription factors in bone formation [12]. Salubrinal is shown to enhance healing of bone wounds and promotes differentiation of osteoblasts [13]. Little is known, however, about its effects on bone resorption, in particular developmental regulation of bone marrow-derived cells. Bone marrow-derived cells contain mesenchymal stem cells (MSCs) and hematopoietic stem cells that give rise to osteoblasts and osteoclasts, respectively [14]. The primary focus of this study is the potential role of salubrinal in the development of bone marrow-derived cells towards mature osteoclasts, as well as its role in development of mesenchymal stem cells and osteoblasts.

Experimental animal models are useful to evaluate therapeutic efficacy of chemical agents. Available osteoporosis models include ovariectomy (OVX) [15,16], tail suspension [17,18], denervation [19,20], a low-calcium diet [21,22], and administration of receptor activator of nuclear factor kappa-B ligand (RANKL) [23-25]. Any animal model may have its advantage and disadvantage. For instance, OVX-induced osteoporosis, which is currently considered as the gold standard for the evaluation of pharmaceuticals for postmenopausal osteoporosis, not only reduces the level of estrogen but also generates surgery-induced injury together with an increase in osteoblast activity. Furthermore, surgical induction of OVX requires consistency in the surgical procedure as well as a minimum of 4 weeks. The tail suspension model not only increases bone resorption but also reduces osteoblast differentiation. In the denervation model, surgery-induced injury is involved. In this study, we evaluated *in vivo* effects of salubrinal using the OVX mice and *in vitro* effects of salubrinal using bone marrow-derived cells isolated from the RANKL-injected mice.

In the RANKL administration model, RANKL is subcutaneously injected for as a short period as 3 days [26]. RANKL is a cytokine belonging to the tumor necrosis factor family. In the immune system, it is involved in dendritic cell maturation, while in the skeletal system it is a ligand for osteoprotegerin (OPG) and functions as a key regulator for osteoclast differentiation and activation [27,28]. RANKL deletion in mice leads to osteopetrosis and a decrease of osteoclasts, while RANKL overproduction is linked to a variety of degenerative bone diseases including osteoporosis and rheumatoid arthritis [29,30].

Focusing on the development of bone marrow-derived cells in the presence and absence of salubrinal, we addressed a pair of questions: Does administration of salubrinal modulate cellular fates and functions of bone marrow-derived cells in favor of prevention of bone loss? If so, are salubrinal's actions more potent to the cells isolated from the osteoporotic RANKL-injected mice than those isolated from the control mice? Because of the anticipated role of salubrinal that is potentially opposite to that of RANKL, we hypothesized that salubrinal is more effective in inhibiting development of osteoclasts and stimulating development of osteoblasts in the cells isolated from the RANKL-injected mice than those from the control mice. To test the hypothesis, we employed assays such as colony-forming unit - granulocyte/macrophages (CFU-GM), colony-forming unit - macrophages (CFU-M), and formation of multi-nucleated osteoclasts in an osteoclast differentiation medium, as well as assays for migration and adhesion of pre-osteoclasts. We also conducted assays for examining colony-forming unit – osteoblasts (CFU-OBL) in an osteoblast differentiation medium. To evaluate salubrinal's effects on expression of nuclear factor of activated T cells c1 (NFATc1), a master transcription factor for osteoclastogenesis, we conducted real-time PCR and Western blot analysis.

Methods

Animals and materials preparation

C57BL/6 female mice (7 weeks of age) were used. Each cage housed four to five mice at the Indiana University Animal Care Facility. They were fed with mouse chow and water *ad libitum*. Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Cytokines were purchased from PeproTech (Rocky Hills, NC, USA) and other chemicals from Sigma (St. Louis, MO, USA) unless otherwise stated. Salubrinal (R&D Systems, Minneapolis, MN, USA) was administered at 1 mg/kg to mice, and at 0.5 to 5 μM to cultured cells for the duration of each experiment.

Ovariectomy

The animal was anesthetized with 1.5% isoflurane at a flow rate of 0.5 to 1.0 L/min. After removing the hair, the skin at the operative sites was cleaned using 70% alcohol and 10% providoneiodine solution. An incision (~20 mm) was made at the midline dorsal skin, and the peritoneal cavity was incised to access the ovaries. After removing the ovaries, the wound was closed by suturing. In 4 weeks after surgery, subcutaneous injection of salubrinal was conducted daily at a dose of 1 mg/kg body weight for 4 weeks. The control OVX mice received an equal volume of vehicle.

RANKL administration for the bone loss model

Soluble recombinant murine RANKL (sRANKL; PeproTech) was injected subcutaneously using a 1 mg/kg dosage in 100 μl PBS at 24 h intervals for 3 days [26]. The same

volume of PBS was injected into vehicle control mice. At 90 min after the final injection, the mice were euthanized. Iliac bones, femora, and tibiae were harvested, and bone marrow-derived cells were collected.

Determination of bone mineral density (BMD) and bone mineral content (BMC)

The BMD (g/cm^2) and BMC (g) of an entire humerus and ulna were determined using peripheral dual energy X-ray absorptiometry (DXA; PIXImus II, Lunar Corp., Madison, WI, USA) and its software (version 1.47).

Colony-forming unit-granulocyte-macrophages (CFU-GM) assay

As previously described, a colony-forming unit-granulocyte-macrophage (CFU-GM) assay was conducted [31-33]. Approximately 2.5x10^4 bone marrow-derived cells were prepared from the vehicle control and RANKL-treated mice and seeded onto a 35-mm gridded dish composed of methylcellulose supplemented with 30 ng/ml murine macrophage-colony stimulating factor (M-CSF), and 20 ng/ml RANKL. Three dosages of salubrinal (1, 2, and 5 μM) were administered, and cells were cultured at 37°C in a 5% CO_2 incubator for 7 days.

Colony-forming unit-macrophage/mononuclear (CFU-M) assay

Using bone marrow mononuclear cells (BMMNCs), a colony-forming unit-macrophage/mononuclear (CFU-M) assay was conducted, as described previously [34-37]. From the vehicle control and RANKL administration mice, approximately 2.5x10^4 bone marrow-derived cells were prepared. Cells were seeded onto a 35-mm gridded dish, which was composed of methylcellulose supplemented with 30 ng/ml M-CSF and 20 ng/ml RANKL. Three dosages of salubrinal (1, 2, and 5 μM) were administered, and cells were cultured at 37°C in a 5% CO_2 incubator for 7 days.

Isolation of bone marrow-derived cells for osteoclast development

Bone marrow-derived cells were collected by flushing the iliac, femur and tibia with Iscove's MEM (Gibco-Invitrogen, Carlsbad, CA, USA) containing 2% fetal bovine serum using a 23-gauge needle, as described previously [34,38]. Low-density gradient centrifugation was used to separate the cells, which were then cultured in α-MEM supplemented with 10% FBS, 30 ng/ml M-CSF, and 20 ng/ml murine receptor activator of nuclear factor kappa-B ligand (RANKL). Culture medium was replaced by α-MEM supplemented with 10% FBS, 30 ng/ml M-CSF, and 60 ng/ml RANKL on the third day, and cells were then grown for an additional 3 days.

Osteoclast differentiation assay

Using bone marrow-derived cells isolated from the vehicle control and RANKL-treated mice with administration of salubrinal (0, 1, 2, and 5 μM) in 96-well plates, an osteoclast differentiation assay was performed, as described previously [34,39,40]. For one experimental condition, salubrinal was applied on day 0 to day 6 (6 days), while in the other experimental condition, it was applied on day 4 to day 6 (3 days). Culture medium was exchanged once on day 4 during the 6-day experiments. A tartrate resistant acid phosphate (TRACP)-staining kit was used according to the manufacturer's instructions to fix and stain adherent cells. TRACP-positive multinuclear cells (> 3 nuclei) were identified as osteoclasts, and their numbers were counted [39]. The osteoclast formation assay was performed at least 3 times using cells isolated independently from different cohorts of mice.

Osteoclast migration assay

Using a transwell assay, migration of osteoclasts was evaluated as described previously with minor modifications [41]. After isolating them from vehicle control and RANKL-treated mice, bone marrow-derived cells (2×10^6/ml) were cultured in M-CSF and RANKL in 6-well plates for 4 days, and then trypsinized in Hank's balanced salt solution. With and without salubrinal (2 μM), the osteoclast precursor cells (1×10^5 cells/well) were loaded onto the upper chamber of transwells and allowed to migrate to the bottom chamber through an 8-μm polycarbonate filter coated with vitronectin (Takara Bio Inc., Otsu, Shigma, Japan). α-MEM consisting of 1% bovine serum albumin (BSA) and 30 ng/ml of M-CSF was in the bottom chamber. After reacting for 6 h, the osteoclast precursor cells in the lower chamber was stained with crystal violet and counted.

Osteoclast adhesion assay

Ninety-six well plates were coated with 5 μg/ml vitronectin in α-MEM supplemented with 30 ng/ml M-CSF and were applied with osteoclast precursors (1×10^5 cells/well) in the presence and absence of salubrinal (2 μM), as described previously [41]. Cells were incubated for 30 min, then washed with PBS three times and fixed with 4% paraformaldehyde at room temperature for 10–15 min. After crystal violet staining, the number of cells adherent to $\alpha_v\beta_3$ integrin was counted.

Osteoblast differentiation assay

Bone marrow-derived cells were plated at 2×10^6/ml in 6-well plates in osteogenic differentiation medium (MesenCult proliferation kit) supplemented with 10 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate to induce osteogenic differentiation, as described previously [39,41,42]. Cells were

cultured for 2 weeks in the presence and absence of salubrinal (0.5 μM), and medium was changed every other day. For alkaline phosphatase (ALP) staining, cells were fixed in citrate-buffered acetone for 30 s, incubated in the alkaline-dye mix for 30 min, and counterstained with Mayer's Hematoxylin for 10 min. Cells were then evaluated microscopically and the intensity of ALP staining was determined.

To evaluate the effects of RANKL administration on multiple developmental stages starting from bone marrow-derived cells to mature osteoclasts, the RANKL-driven alterations in CFU-GM, CFU-M, osteoclast formation, migration, and adhesion were determined as fold-changes of the RANKL-injected mice to the vehicle control mice. Furthermore, to quantify efficacy of salubrinal in various developmental stages in osteoclastogenesis, the degree of suppression was measured with reduction ratios between the samples treated with and without 2 μM salubrinal.

Expression analysis of NFATc1 in bone marrow-derived cells and RAW264.7 pre-osteoclast cells

For Western blot analysis, RAW264.7 mouse monocyte/macrophage cells (ATCC, Manassas, VA, USA) were grown in α–MEM containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin, and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA). To induce osteoclastogenesis, 20 ng/ml of RANKL was administered. Bone marrow-derived cells or RAW264.7 cells were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer, containing protease inhibitors (Santa Cruz Biotechd, Santa Cruz, CA, USA) and phosphatase inhibitos (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10% SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Antibodies specific to NFATc1 (Santa Cruz), and β-actin (Sigma) were employed. Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific). The bands were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and their intensities were quantified using Image J.

In quantitative PCR, total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA) and reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed using Power SYBR green PCR master mix kits (Applied Biosystems). The PCR primers were: NFATc1 (5′-GGT GCT GTC TGG CCA TAA CT-3′; and 5′-GCG GAA AGG TGG TAT CTC AA-3′), tartrate-resistant acid phosphatase (TRACP) (5′- TCC TGG CTC AAA AAG CAG TT -3′; and 5′- ACA TAG CCC ACA CCG TTC TC -3′); and GAPDH (5′-TGC ACC ACC AAC TGC TTA G-3′; and 5′-GGA TGC AGG GAT GAT GTT C-3′), in which GAPDH was used for internal control. Since TRACP is highly expressed in osteoclasts, we used its mRNA expression level as a marker for development of osteoclasts. The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a ratio of $S_{treated}/S_{control}$, where $S_{treated}$ is the mRNA level for the cells treated with salubrinal, and $S_{treated}$ is the mRNA level for control cells.

Statistical analysis

The data were expressed as mean ± standard error of mean (SEM). Student's t-test was conducted for two-group comparisons. For many-group comparisons, one-way ANOVA was used, followed by a post-hoc test using Fisher's protected least significant difference. All comparisons were two-tailed, and statistical significance was assumed at $p < 0.05$. The asterisks (*, **, and ***) represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Results

Evaluation of BMD and BMC of the OVX mice and RANKL-injected mice

Four-week daily administration of salubrinal at a dose of 1 mg/kg to the OVX mice significantly elevated both BMD and BMC of a whole body (Figure 1A-B). Three-day administration of RANKL at a dose of 1 mg/kg, however, significantly decreased BMD and BMC of the humerus and ulna (N = 6; both $p < 0.05$) (Figure 1C-D). Using the RANKL-injected mice, bones from the Iliac, femora, and tibiae were harvested. Bone marrow-derived cells were collected from those bones for examining the effects of salubrinal on developments of osteoclasts and osteoblasts.

Reduction in the number of CFU-GM by salubrinal in a dosage-dependent manner

To determine the effects of salubrinal on the proliferation of osteoclast progenitors, the CFU-GM assay was conducted using bone marrow-derived cells isolated from the RANKL-injected mice. Salubrinal at 1, 2, and 5 μM reduced the total number of CFU-GM in the femur in a dosage-dependent manner ($p < 0.05$ for 1 μM salubrinal; $p < 0.01$ for 2 μM; and $p < 0.001$ for 2 & 5 μM) in the RANKL-injected mice (Figure 2A). The CFU-GM numbers were 37,177 ± 1,919 (vehicle control) and 53,213 ± 3,545 (RANKL administration, $p < 0.001$) (Figure 2B). The CFU-GM numbers were reduced by administration of salubrinal at 2 μM for 7 days by 28.5% ($p < 0.001$) in vehicle control and 30.8% ($p < 0.001$) in the RANKL-injected mice.

Reduction in the number of CFU-M by salubrinal in a dosage –dependent manner

To determine the effects of salubrinal on the population of osteoclast progenitors, the CFU-M assay was performed using bone marrow-derived cells isolated from the

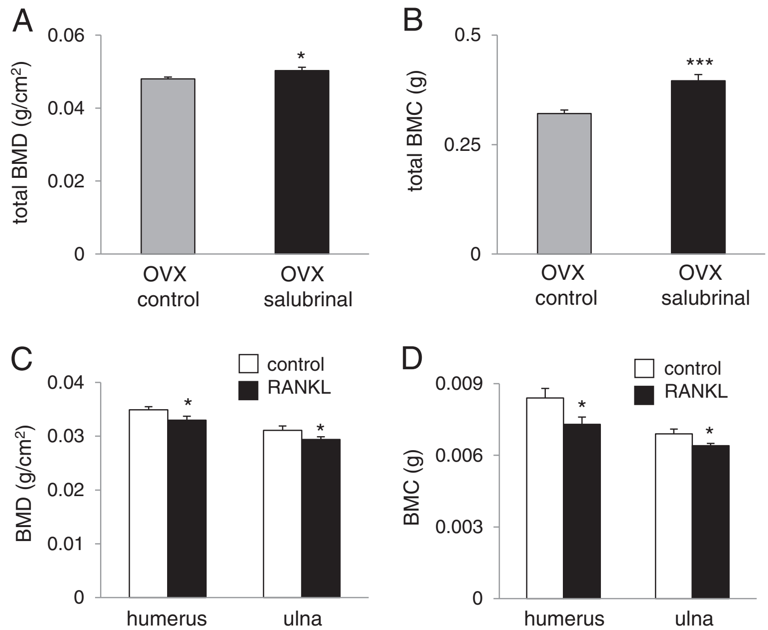

Figure 1 Determination of BMD and BMC in the OVX mice and RANKL-injected mice. A: Increase in BMD (g/cm^2) of the OVX mice by salubrinal (N = 8). **B**: Increase in BMC (g) of the OVX mice by salubrinal (N = 8). **C**: Decrease in BMD (g/cm^2) of the humerus and ulna of the RANKL-injected mice (N = 6). **D**: Decrease in BMC (g) of the humerus and ulna (N = 6).

RANKL-injected mice. Consistent with the CFU-M numbers, administration of salubrinal at 1, 2, and 5 μM reduced the total number of CFU-M in the femur in a dosage-dependent manner (all $p < 0.001$ in three dosages) (Figure 3A). The CFU-M numbers were 10,602 ± 396 (vehicle control) and 18,648 ± 760 (RANKL administration, $p < 0.001$) (Figure 3B). Administration of salubrinal at 2 μM for 7 days, for instance, reduced the CFU-M number by 41.2% ($p < 0.001$) in vehicle control and 43.1% ($p < 0.001$) in the RANKL-injected mice.

Suppression of osteoclast differentiation by salubrinal in a dosage– and time-dependent manner

Compared to the bone marrow-derived cells isolated from the vehicle control, the cells from the RANKL-injected mice exhibited an increase in the surface area occupied by multi-nucleated osteoclasts (24.8 ± 1.0% in vehicle control, and 36.5 ± 1.3% in RANKL administration) (Figure 4A). A series of images show that the process of osteoclast fusion was accelerated by administration of salubrinal. To evaluate the effects of salubrinal, three dosages of salubrinal (1, 2, and 5μM) were applied. In the cultures salubrinal was applied on day 0 for 6 days, administration of salubrinal resulted in a significant decrease in the surface area covered by multi-nucleated osteoclasts for vehicle control (all $p < 0.001$) and RANKL administration (all $p < 0.001$) (Figure 4A). In the cultures salubrinal was applied on day 3 for 4 days, the reduction of the area was also observed (all $p < 0.001$) (Figure 4B). A series of images indicate that the cellular fusion was reduced by salubrinal administration in a time-and dose-dependent manner.

To further evaluate potential effects of the period of salubrinal administration on osteoclast formation, we compared the results of two sets of experiments in which salubrinal at 2 μM was administered from days 0 to 6, and days 4 to 6. The result revealed that salubrinal administration at day 0 presented larger reduction in osteoclast formation than that at day 4 in the vehicle control and RANKL-injected groups (both $p < 0.001$) (Figure 4C).

Suppression of migration and adhesion of pre-osteoclasts by salubrinal

Pre-osteoclast cells isolated from the RANKL-injected mice were more migratory (304.1 ± 12.2 cells) than those from the vehicle control (190.4 ± 5.9 cells, $p < 0.001$), and the RANKL-driven increase was 37.4% (Figure 5A). However, salubrinal suppressed the amount of migration by 33.0% in vehicle control ($p < 0.001$) and by 53.2% in RANKL administration ($p < 0.001$). In the M-CSF mediated adhesion assay to $\alpha_V\beta_3$, the cells isolated from the RANKL-injected mice presented an increase in adhesion by 59.8% (142.5 ± 3.9 cells) over those from the vehicle control (57.3 ± 1.8 cells, $p < 0.001$) (Figure 5B). Administration of salubrinal presented significant reduction in cell adhesion by 32.4% in vehicle control ($p < 0.001$) and by 53.7% in RANKL administration ($p < 0.001$).

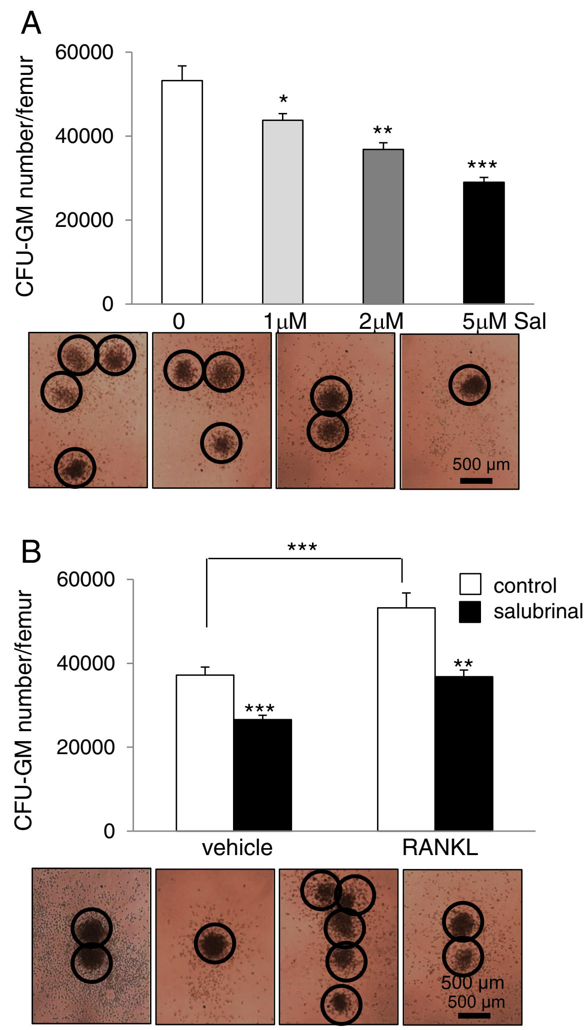

Figure 2 Effects of salubrinal on colony-forming unit-granulocyte-macrophage (CFU-GM). Approximately 2.5x10^4 bone marrow-derived cells were prepared and seeded onto a 35-mm gridded dish supplemented with 30 ng/ml murine M-CSF and 20 ng/ml RANKL. Three dosages of salubrinal (1, 2, and 5 μM) were administered, and cells were cultured for 7 days. **A**: Salubrinal-induced reduction in CFU-GM numbers in the RANKL-injected mice using three dosage of salubrinal. The images exhibit the 4 different CFU-GM cultures, in which the circles indicate the colonies. **B**: Comparison of CFU-GM numbers in the vehicle control and RANKL-injected mice with and without *in vitro* administration of salubrinal. The representative microphotographs are shown, displaying 4 CFU-GM cultures with colonies in circle. Bar = 500 μm.

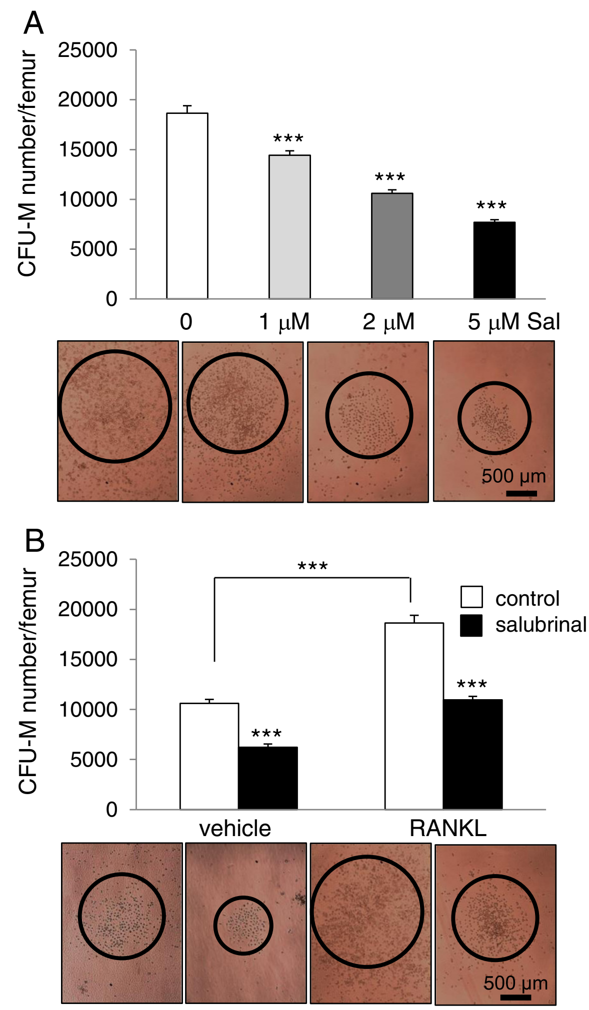

Figure 3 Effects of salubrinal on colony-forming unit-macrophage/monocyte (CFU-M). Approximately 2.5x10^4 bone marrow mononuclear cells were prepared and seeded onto a 35-mm gridded dish supplemented with 30 ng/ml M-CSF and 20 ng/ml RANKL. Three dosages of salubrinal (1, 2, and 5 μM) were administered and cells were cultured for 7 days. **A**: Salubrinal-induced reduction in CFU-M numbers in the RANKL-injected mice using three dosage of salubrinal. The images exhibit the 4 different CFU-M cultures, in which the circles indicate the colonies. **B**: Comparison of CFU-M numbers in the vehicle control and RANKL-injected mice with and without *in vitro* administration of salubrinal. The representative microphotographs are shown, displaying 4 CFU-M cultures with colonies in circle. Bar = 500 μm.

Promotion of osteoblast differentiation by salubrinal

In the CFU-OBL assay, a significant increase in the number of ALP positive cells was detected by administration of salubrinal. Without salubrinal, the percentage of ALP-positive cells was 18.3 ± 2.3% in vehicle control and 20.4 ± 2.0% in RANKL administration ($p < 0.001$) (Figure 6). Administration of salubrinal at 0.5 μM increased the percentage of ALP-positive cells to 23.5 ± 1.1% in vehicle control ($p < 0.05$) and 28.8 ± 2.3% in RANKL administration ($p < 0.01$) (Figure 6).

Downregulation of NFATc1 by salubrinal in bone marrow-derived cells and RAW264.7 pre-osteoclast cells

Bone marrow-derived cells were incubated with RANKL in the presence and absence of salubrinal. Incubation with 20 ng/ml RANKL markedly increased the level of NFATc1, a master transcription factor for development of osteoclasts, and administration of 1 μM salubrinal

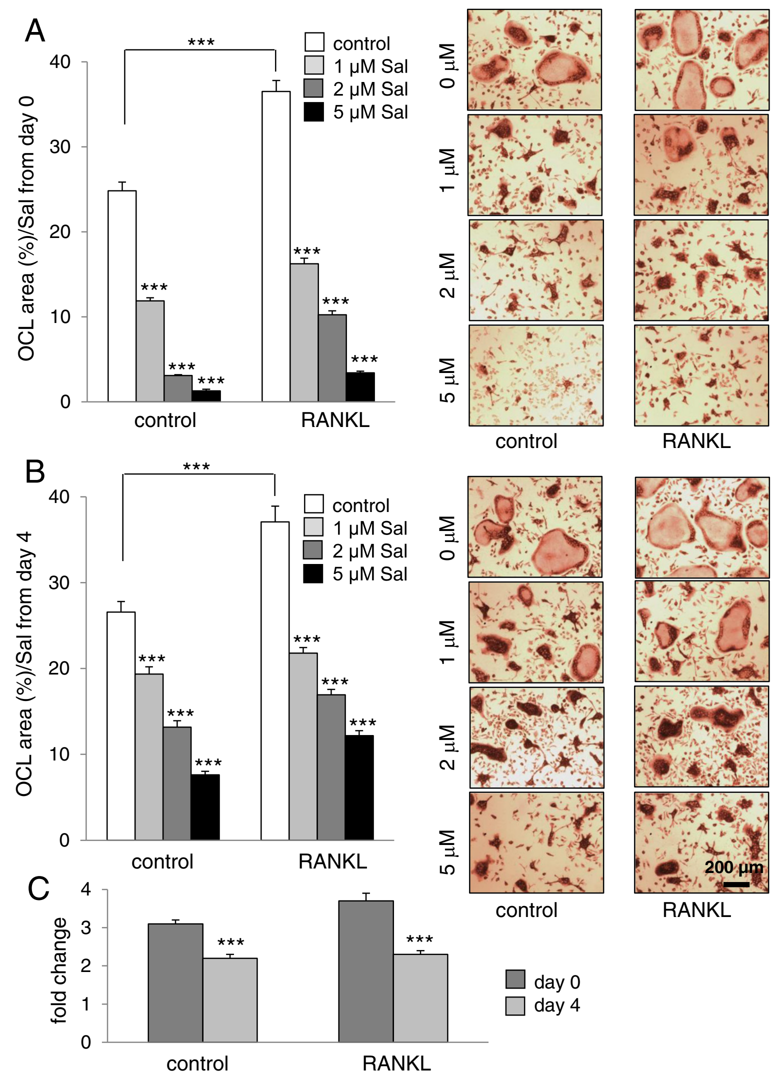

Figure 4 Effects of administration of salubrinal on multi-nucleated osteoclast formation. Using bone marrow-derived cells isolated from the vehicle control and RANKL-treated mice, an osteoclast differentiation assay was performed. The culture medium was exchanged once on day 4 during the 6-day experiments. TRACP-positive multinuclear cells (> 3 nuclei) were identified as osteoclasts. The areas covered by multi-nucleated osteoclasts are quantified in response to 3 doses of salubrinal (1, 2, and 5 μM). The microphotographs represent the two groups of osteoclast cultures (vehicle control and RANKL administration) with TRACP staining. Bar = 200 μm. **A**: Area covered by multi-nucleated osteoclasts in response to *in vitro* administration of salubrinal from day 0 to day 6 (6 days). **B**: Area covered by multi-nucleated osteoclasts in response to *in vitro* administration of salubrinal from day 4 to day 6 (3 days). **C**: Fold change in response to 2 μM salubrinal.

reduced the RANKL-driven increase in NFATc1 by 24% (Figure 7A). To further evaluate the effects of salubrinal, we employed RAW264.7 pre-osteoclast cells. Administration of 20 ng/ml RANKL elevated the level of NFATc1, and in response to 1–20 μM salubrinal the RANKL-induced elevation of NFATc1 was reduced in a dose dependent fashion (Figure 7B). Furthermore, the mRNA levels of NFATc1 and TRACP were increased by RANKL, and their elevation was suppressed by administration of salubrinal (Figure 7C).

Discussion

The present study presents the beneficial effect of *in vivo* administration of salubrinal on BMD and BMC of the OVX mice, and *in vitro* effects on the culture of bone marrow-derived cells isolated from the RANKL-injected and control mice. In the osteoclast assays of CFU-GM, CFU-M, and formation of multi-nucleation, salubrinal significantly reduced the numbers of osteoclastic colonies and cells isolated from both the vehicle control and RANKL-injected mice. In the two sets of maturation

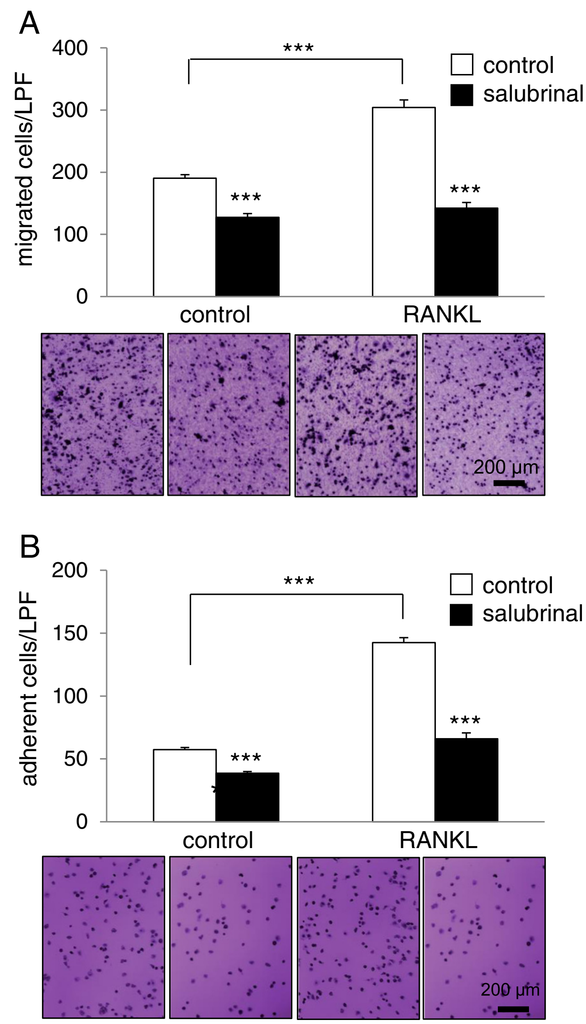

Figure 5 Effects of salubrinal on migration and adhesion of pre-osteoclasts. Bone marrow-derived cells (2 × 10^6/ml) were cultured in M-CSF and RANKL in 6-well plates for 4 days to obtain pre-osteoclasts used for the migration and adhesion assays. **A**: Number of migratory cells. Osteoclast precursor cells (1 × 10^5 cells/well) were loaded onto the upper chamber of transwells in the presence and absence of 2 μM salubrinal. The bottom chamber was filled with α-MEM consisting of 1% BSA and 30 ng/ml of M-CSF, and cells were allowed to migrate to the bottom chamber through an 8-μm polycarbonate filter coated with vitronectin. After reacting for 6 h, the cells in the lower chamber was stained with crystal violet and counted. The images display 2 pairs of osteoclast cultures. Bar = 200 μm. **B**: Number of adherent cells. Ninety-six well plates were coated with 5 μg/ml vitronectin and filled with α-MEM supplemented with 30 ng/ml M-CSF. Approximately 1 × 10^5 osteoclast precursor cells were cultured per well in the presence and absence of 2 μM salubrinal for 30 min. Cells were stained with crystal violet and the number of cells adherent to $\alpha_v\beta_3$ integrin was counted. Bar = 200 μm.

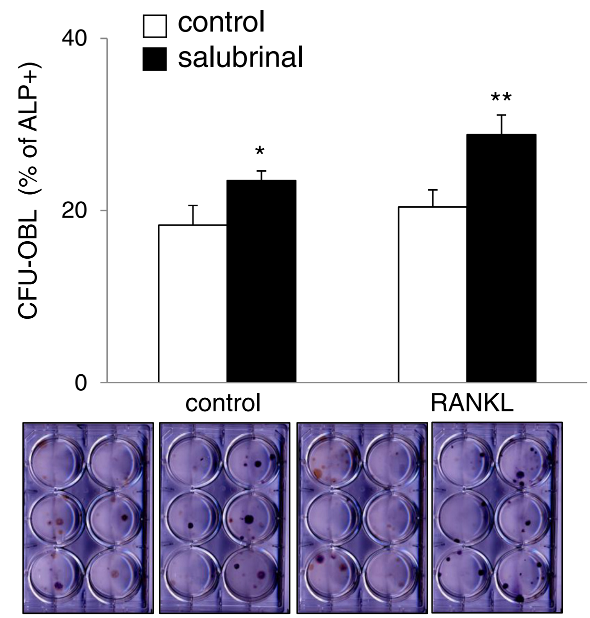

Figure 6 Enhanced osteoblast development by salubrinal in the CFU-OBL assay. Bone marrow-derived cells were plated at 2 × 10^6/ml in 6-well plates in osteogenic differentiation medium supplemented with 10 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate. Cells were cultured for 2 weeks in the presence and absence of 0.5 μM salubrinal, and medium was changed every other day. For alkaline phosphatase (ALP) staining, cells were fixed in citrate-buffered acetone for 30 s, incubated in the alkaline-dye mix for 30 min, and counterstained with Mayer's Hematoxylin for 10 min. Cells were then evaluated microscopically and the intensity of ALP staining was determined. The images display 2 pairs of osteoblast cultures.

assays, in which salubrinal was applied from day 0 to 6 and from day 4 to 6, it suppressed both the early and late stages of osteoclastogenesis. This suppressive effect was larger in the cells isolated from the RANKL-injected mice than the vehicle control mice. In addition to attenuating osteoclastogenesis, salubrinal was able to reduce adhesion and migration of osteoclasts. Furthermore, it increased the number of CFU-OBL colonies suggesting that it not only inhibits development of osteoclasts but also promotes development of osteoblasts. Quantitative PCR and Western blot analysis revealed that the mRNA and protein levels of NFATc1 were elevated by RANKL, and this elevation was suppressed by administration of salubrinal in a dose dependent fashion.

In evaluating the effects of salubrinal on fates of HSCs and MSCs in bone marrow-derived cells, we employed the recently developed RANKL administration model of osteoporosis. An advantage of this RANKL administration model includes a short period (3 days in this study) for induction of osteoclastogenesis, and activation of multiple steps in the development of osteoclasts. In the RANK/RANKL/OPG signaling pathway, RANKL regulates not only development of osteoclasts but also their activation and survival [43]. RANKL is expressed in bone, bone marrow, and lymphoid tissues including spleen that houses osteoclast precursor cells as macrophages [44]. The RANKL administration model provided a platform to

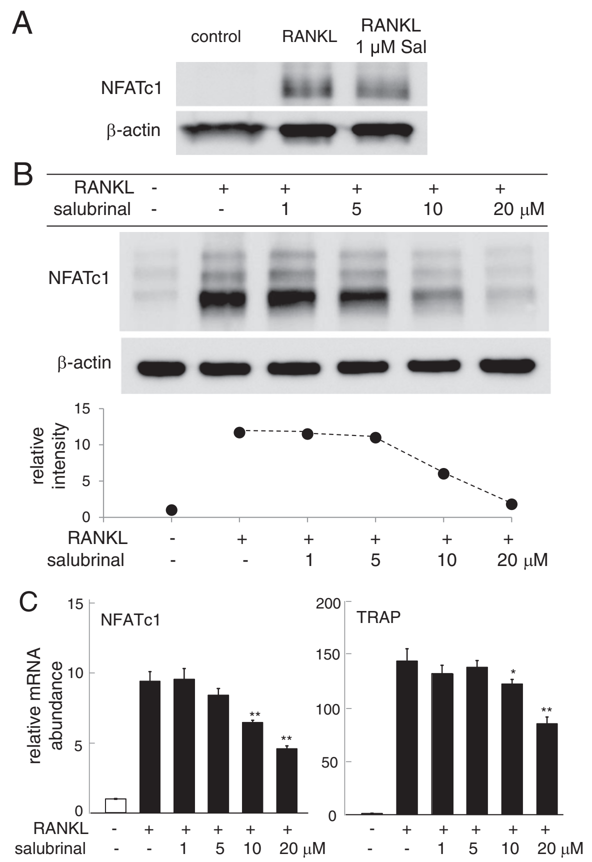

Figure 7 Suppression of RANKL-induced expression of NFATc1 and TRACP by salubrinal in RAW264.7 cells. A: Salubrinal-driven suppression of NFATc1 expression in RANKL-stimulated bone marrow-derived cells. **B**: Dose dependent suppression of NFATc1 in response to 1 – 20 μM salubrinal. β-actin was used as a loading control. **C**: Relative mRNA levels of NFATc1 and TRACP in response to 1 – 20 μM salubrinal. The mRNA levels are normalized by the mRNA level of the sample without salubrinal in the absence of RANKL stimulation.

evaluate efficacy of salubrinal as a potential therapeutic agent for preventing osteoclastogenesis and bone resorption. Besides bone resorption, however, RANKL is involved in multiple functions in the immune system such as proliferation of T cells and inhibition of apoptosis of dendritic cells [45]. It is reported that overproduction of RANKL induces inflammatory bone disorders [46,47]. Thus, the results from any animal model including the RANKL administration model should be confirmed by other animal models and eventually clinical trial.

The regulatory mechanism of salubrinal's action on osteoclastogenesis is not well understood. Salubrinal is known as an inhibitor of serine/threonine-protein phosphatase PP1 and it elevates the phosphorylation level of eIF2α (eIF2α-p) [48]. The level of eIF2α-p is upregulated in response to various stresses including viral infection, nutrient deprivation, radiation, and stress to the endoplasmic reticulum [49]. To cope with these cellular insults and reduce apoptosis, the elevated eIF2α-p in general lowers ribosome's efficiency of protein synthesis except for a group of proteins such as ATF4. Applications of salubrinal have been reported to reduce stress induced apoptosis [50]. We have previously shown that partial silencing of eIF2α by RNA interference reduces salubrinal-driven downregulation of NFATc1 in RAW264.7 cells [51], and the results in this study indicate that mRNA and protein expression of NFATc1 is downregulated by salubrinal. NFATc1 is a member of the NFAT transcription factor family and a master transcription factor for osteoclast development. It is reported that NFATc1-deficient embryonic stem cells are unable to differentiate into osteoclasts [52]. He et al. has recently shown that NFATc1 expression is regulated at a translational stage in bone marrow macrophage cells, and a phosphorylation mutant plasmid for eIF2α restored RANKL-induced NFATc1 expression [53]. MafB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), IRF8 (interferon regulatory factor 8), and Bcl6 (V cell lymphoma) have been mentioned as inhibitors of NFATc1 [54-56]. Further analysis is necessary for identification of the mechanism of salubrinal's action on NFATc1, which is possibly regulated by eIF2α alone or any other mediators.

Conclusions

It is premature to draw any conclusion on development of a potential therapeutic agent for treatment of osteoporosis, but salubrinal possesses several unique features. First, it is a small synthetic chemical agent, which can be taken as an oral pill. Second, it has a dual role of stimulation of bone formation and attenuation of bone resorption. Third, its effects are stronger in the cells isolated from the osteoporotic RANKL-injected mice than those from the control mice. Fourth, it presents dose dependent efficacy in preventing osteoclastogenesis throughout a developmental stage including proliferation, multi-nucleation, and maturation, as well as migration and adhesion. The results herein support the possibility of preventing bone loss through salubrinal-driven regulation of bone marrow-derived cells.

Abbreviations

OVX: Ovariectomy; BMD: Bone mineral density; BMC: Bone mineral content; eIF2α: Eukaryotic translation initiation factor 2 alpha; eIF2α-p: Phosphorylated eIF2α; NFκB: Nuclear factor kappa B; ATF4: Activating transcription factor 4; NFATc1: Nuclear factor of activated T cells c1; OPG: Osteoprotegerin; RANKL: A Receptor activator of nuclear factor kappa-B ligand; M-CSF: Murine macrophage-colony stimulating factor; BMMNCs: Bone marrow mononuclear cells; MSCs: Mesenchymal stem cells; CFU-M: Colony forming-unit macrophages; CFU-GM: Colony forming-unit granulocyte/macrophages; CFU-OBL: Colony-forming unit – osteoblasts; ALP: Alkaline phosphatase; TRACP: Tartrate resistant acid phosphate; BSA: Bovine serum albumin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HY participated in experimental designs, and drafted a manuscript. KH conducted molecular experiments, performed data collection and analysis. AC and TD conducted animal experiments. NT and AA assisted data collection. PZ participated in experimental designs, performed animal and cell experiments, conducted data collection and interpretation, and drafted a manuscript. PZ accepted responsibility for integrity of data analysis. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha

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Abstract In response to various stresses including viral infection, nutrient deprivation, and stress to the endoplasmic reticulum, eukaryotic translation initiation factor 2 alpha (eIF2α) is phosphorylated to cope with stress induced apoptosis. Although bone cells are sensitive to environmental stresses that alter the phosphorylation level of eIF2α, little is known about the role of eIF2α mediated signaling during the development of bone-resorbing osteoclasts. Using two chemical agents (salubrinal and guanabenz) that selectively inhibit de-phosphorylation of eIF2α, we evaluated the effects of phosphorylation of eIF2α on osteoclastogenesis of RAW264.7 pre-osteoclasts as well as development of MC3T3 E1 osteoblast-like cells. The result showed that salubrinal and guanabenz stimulated matrix deposition of osteoblasts through upregulation of activating transcription factor 4 (ATF4). The result also revealed that these agents reduced expression of the nuclear factor of activated T cells c1 (NFATc1) and inhibited differentiation of RAW264.7 cells to multinucleated osteoclasts. Partial silencing of eIF2α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. Collectively, we demonstrated that the elevated phosphorylation level of eIF2α not only stimulates osteoblastogenesis but also inhibit osteoclastogenesis through regulation of ATF4 and NFATc1. The results suggest that eIF2α-mediated signaling might provide a novel therapeutic target for preventing bone loss in osteoporosis.

Keywords Osteoclasts · Salubrinal · Guanabenz · eIF2α · NFATc1

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Introduction

Osteoblasts and osteoclasts are the two major types of bone cells in bone remodeling. Osteoblasts are bone-forming cells originated from mesenchymal stem cells, while osteoclasts are bone-resorbing cells derived from hematopoietic stem cells. These two types of cells orchestrate a complex remodeling process, in which mineralized bone matrix is degraded by osteoclasts and newly formed by osteoblasts [1, 2]. In order to maintain proper bone mass, exercise and calcium rich diets are recommended. However, a failure of the coordinated action such as in osteoporosis, which is a common form of bone loss prevailing among postmenopausal women, increases risk of bone fracture [3]. In order to develop therapeutic drugs for treatment of osteoporosis, an understanding of signaling pathways that govern osteoclastogenesis—development of pre-osteoclasts (monocyte/macrophage) to multi-nucleated osteoclasts—is required. In this paper, we examined a signaling pathway for osteoclastogenesis that is mediated by eukaryotic translation initiation factor 2 alpha (eIF2α).

A protein complex, eIF2, is a heterotrimer essential for protein synthesis, and eIF2α is one of its major components together with eIF2β and eIF2γ [4]. In response to various stresses such as oxidation, radiation, and stress to the

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endoplasmic reticulum that potentially lead to cellular apoptosis, a serine residue of eIF2α is phosphorylated. This action would initiate a pro-survival program by lowering general translation efficiency except for a group of genes that includes activating transcription factor 4 (ATF4) [5]. The ATF4 is a transcription factor critical for osteoblastogenesis and bone formation [6]. In osteoblasts elevation of phosphorylated eIF2α (p-eIF2α) is reported to stimulate the expression of ATF4 [7, 8]. Little is known, however, about potential effects of p-eIF2α on development of osteoclasts.

Herein we addressed a question: Does elevation of p-eIF2α alter cellular fates of pre-osteoclasts? Osteoblasts and osteoclasts extensively interact through molecular pathways including RANK (receptor activator of nuclear factor kappa-B)/RANKL (RANK ligand)/OPG (osteoprotegerin) signaling [9, 10] and Wnt signaling [11]. Therefore, osteoclastogenesis is potentially regulated by signaling molecules that also affect osteoblastogenesis. Furthermore, osteoclastogenesis is influenced by various stresses such as estrogen deficiency and disuse or unloading [12]. Since elevation of p-eIF2α can provide stress-relieving effects on osteoblasts, we hypothesized that elevation of p-eIF2α suppresses differentiation of pre-osteoclasts to multi-nucleated osteoclasts.

In this study, we employed two chemical agents (salubrinal and guanabenz) and examined the effects of elevated p-eIF2α on osteoclastogenesis. These two agents selectively inhibit de-phosphorylation of p-eIF2α by interacting with protein phosphatase 1, PP1 [13, 14]. The signaling pathway, mediated by eIF2α, is not directly linked to known agents for osteoclastogenesis such as calcium binding agents and RANKL. Currently, the most common medications, prescribed for preventing bone loss in patients with osteoporosis, are bisphosphonates. Bisphosphonates preferentially bind to calcium in bone and induce apoptosis of osteoclasts [15]. Other medications using neutralizing antibodies targeted to RANKL would block osteoclastogenesis by mimicking OPG's binding to RANKL [16]. The RANKL is a cytokine belonging to the tumor necrosis factor family, and is involved in T cell-dependent immune responses as well as differentiation and activation of osteoclasts [9, 10]. To our knowledge, no therapeutic agents for osteoporosis have been targeted to eIF2α-mediated signaling.

We employed MC3T3 E1 osteoblast-like cells [17] and RAW264.7 cells [18] to evaluate osteoblastogenesis and osteoclastogenesis, respectively. In the presence and absence of salubrinal and guanabenz, MC3T3 E1 cells were cultured in an osteogenic medium for evaluation of matrix deposition, while RAW264.7 cells were cultured in an osteoclast differentiation medium for evaluation of multi-nucleation. Alizarin Red S staining was performed to evaluate osteoblast mineralization for MC3T3 E1 cells, and TRAP staining was conducted to determine multi-nucleated osteoclasts proliferation for RAW264.7 cells. To analyze molecular signaling pathways, quantitative real-time PCR and Western blot analysis were conducted. The mRNA levels of ATF4, osteocalcin, c-Fos [19], tartrate-resistant acid phosphatase (TRAP) [20], and osteoclast-associated receptor (OSCAR) [21] were determined. The protein expression levels of eIF2α, ATF4, and nuclear factor of activated T cells c1 (NFATc1) [22] were also determined. The NFATc1 is a transcription factor, which is critically important for development and activation of osteoclasts in response to RANKL. The RNA interference using siRNA specific to ATF4 and eIF2α was conducted to evaluate the role of ATF4 in osteoblastogenesis and eIF2α in osteoclastogenesis.

Materials and methods

Cell culture

The MC3T3 E1 mouse osteoblast-like cells (clone 14—MC3T3 E1-14; and no clonal cells in supplementary figures), and RAW264.7 mouse pre-osteoclast (monocyte/macrophage) cells were cultured in αMEM containing 10 % fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37 °C and 5 % CO_2 in a humidified incubator. Cell mortality and live cell numbers were determined 24 h after the treatment with 20 ng/ml RANKL (PeproTech, Rocky Hills, NC, USA) in response to 0.1–20 μM salubrinal or 1–20 μM guanabenz acetate (Tocris Bioscience, Ellisville, MO, USA). Cells were stained with trypan blue and the numbers of live and dead cells were counted using a hemacytometer.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of ATF4, Osteocalcin (OCN), NFATc1, c-Fos, tartrate-resistant acid phosphatase (TRAP), and osteoclast-associated receptor (OSCAR) with the PCR primers listed in Table 1. The GAPDH was used for internal control. The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a

Table 1 Real-time PCR primers used in this study

Target	Forward primer	Backward primer
ATF4	5′-TGGCGAGTGTAAGGAGCTAGAAA-3′	5′-TCTTCCCCCTTGCCTTACG-3′
OCN	5′-CCGGGAGCAGTGTGAGCTTA-3′	5′-AGGCGGTCTTCAAGCCATACT-3′
NFATc1	5′-GGTGCTGTCTGGCCATAACT-3′	5′-GCGGAAAGGTGGTATCTCAA-3′
c-Fos	5′-AGGCCCAGTGGCTCAGAGA-3′	5′-CCAGTCTGCTGCATAGAAGGAA-3′
TRAP	5′-TCCTGGCTCAAAAAGCAGTT-3′	5′-ACATAGCCCACACCGTTCTC-3′
OSCAR	5′-ACACACACACCTGGCACCTA-3′	5′-GAGACCATCAAAGGCAGAGC-3′
GAPDH	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′

ratio of $S_{treated}/S_{control}$, where $S_{treated}$ is the mRNA level for the cells treated with chemical agents, and $S_{control}$ is the mRNA level for control cells [23].

Western immunoblotting

Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10–15 % SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against ATF4, NFATc1 (Santa Cruz), p-eIF2α (Thermo Scientific, Waltham, MA, USA), eIF2α, caspase 3, cleaved caspase 3, p38 and p-p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p-ERK, nuclear factor kappa B (NFκB) p65 and p-NFκB p65 (Cell Signaling), and β-actin (Sigma). Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

Knockdown of ATF4 and eIF2α by siRNA

Cells were treated with siRNA specific to ATF4 and eIF2α (Life Technologies). Selected target sequences for knockdown of ATF4 and eIF2α were: ATF4, 5′-GCU GCU UAC AUU ACU CUA A-3′; and eIF2α, 5′-CGG UCA AAA UUC GAG CAG A-3′. As a nonspecific control, a negative siRNA (Silencer Select #1, Life Technologies) was used. Cells were transiently transfected with siRNA for ATF4, eIF2α or control in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing was assessed with immunoblotting or quantitative PCR 48 h after transfection.

Mineralization assay

Mineralization of extracellular matrix was assayed by Alizarin Red S staining. The MC3T3-E1 cells were plated in 6-well plates. When cells were confluent, 50 μg/ml of ascorbic acid (Wako Chemicals, Richmond, VA, USA) and 5 mM β-glycerophosphate (Sigma) were added. The medium was changed every other day, and staining was conducted after 3 weeks. Cells were washed with PBS twice and fixed with 60 % isopropanol for 1 min at room temperature, followed by rehydration with distilled water for 3 min at room temperature. They were stained with 1 % Alizarin red S (Sigma) for 3 min and washed with distilled water.

Osteoclastogenesis in vitro and TRAP (Tartrate-resistant acid phosphatase) staining

The RAW264.7 cells were plated at a density of 5×10^3/cm^2 into a 12-well or a 60 mm dish, and cultured with 20 ng/ml RANKL in the presence and absence of salubrinal or guanabenz. The culture medium was replaced every 2 days. After 5 days of culture, the cells were stained for TRAP staining using an acid phosphatase leukocyte kit (Sigma). The number of TRAP-positive cells containing three or more nuclei was determined.

Statistical analysis

Three or four-independent experiments were conducted and data were expressed as mean ± SD. For comparison among multiple samples, ANOVA followed by post hoc tests was conducted. Statistical significance was evaluated at $p < 0.05$. The single and double asterisks and daggers indicate $p < 0.05$ and $p < 0.01$. To determine intensities in immunoblotting and areas of Alizarin red S staining, images were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and quantified using Image J.

Results

Enhanced mineralization of MC3T3 E1-14 cells by salubrinal

Prior to examining the effects of salubrinal on osteoclastogenesis, we tested its effects on the development of osteoblasts focusing on cell viability, phosphorylation of eIF2α (p-eIF2α), expression of ATF4 and osteocalcin, and matrix mineralization. Administration of 5–20 μM salubrinal to MC3T3 E1-14 cells did not increase cell mortality or inhibit cell proliferation (Fig. 1a). Unlike application of 10 nM thapsigargin, which is a stress inducer to the endoplasmic reticulum that elevates p-eIF2α, incubation with 10 μM salubrinal for 24 h did not elevate the expression level of cleaved caspase 3 (Fig. 1b). After 3-week incubation in an osteogenic medium, Alizarin red S staining area showed that salubrinal enhanced mineralization of MC3T3 E1-14 cells in a dose dependent manner (Fig. 1c). The enhanced mineralization was also observed in non-clonal MC3T3 E1 cells (Supplementary Fig. S1).

ATF4-mediated elevation of osteocalcin mRNA in MC3T3 E1-14 cells

Salubrinal is an inhibitor of de-phosphorylation of eIF2α. Administration of 5 μM salubrinal to MC3T3 E1-14 cells elevated phosphorylation of eIF2α, followed by an increase in ATF4 expression (Fig. 2a). Furthermore, the level of osteocalcin mRNA was increased 3.3 ± 0.5 fold (24 h) and 3.3 ± 0.3 fold (32 h) (Fig. 2b). When expression of ATF4 was significantly reduced by RNA interference (Fig. 2c, d), however, salubrinal-driven elevation of the osteocalcin mRNA level was suppressed (Fig. 2e). Non-clonal MC3T3 E1 cells also presented elevation of p-eIF2α and ATF4, together with an increase in the mRNA levels of ATF4 and osteocalcin (Supplementary Fig. S2). In addition, administration of guanabenz to MC3T3 E1-14 elevated the mRNA level of osteocalcin in a dose dependent manner, consistent with an increase in p-eIF2α and ATF4 (Supplementary Fig. S3).

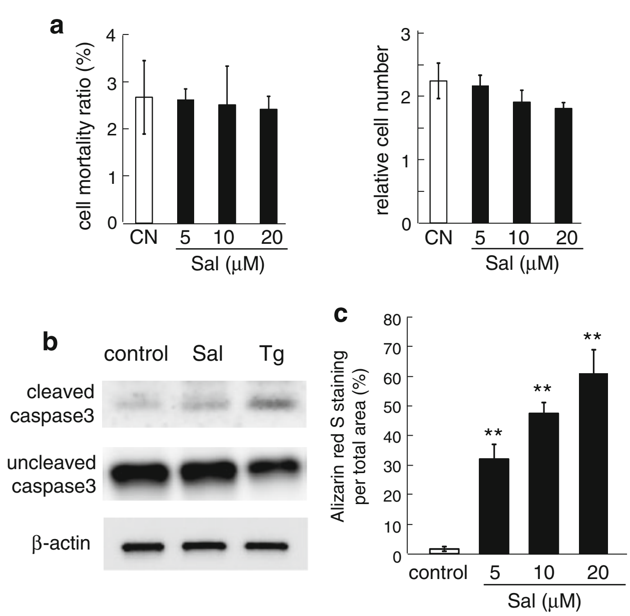

Fig. 1 Osteogenic effects of salubrinal on MC3T3 E1 (clone 14) osteoblast cells. *CN* control, *Sal* salubrinal, and *Tg* thapsigargin. The *double asterisk* indicates $p < 0.01$ in comparison to CN. **a** Cell mortality ratio and relative cell numbers. **b** No activation of cleaved caspase 3 by salubrinal. **c** Alizarin red S staining area in response to 5, 10, and 20 μM salubrinal

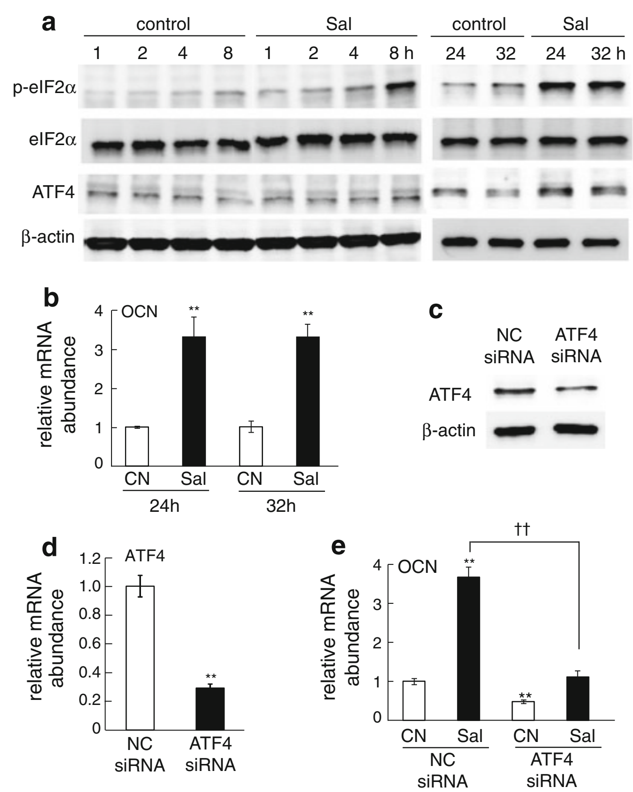

Fig. 2 Upregulation of p-eIF2α, ATF4 and osteocalcin by salubrinal in MC3T3 E1 (clone 14) osteoblast cells in response to 5 μM salubrinal. *CN* control, *Sal* salubrinal, and *NC* non-specific control siRNA. The *double asterisk* indicates $p < 0.01$ in comparison to CN or NC. The *double dagger* indicates with $p < 0.01$ in comparison to the salubrinal-treated NC siRNA cells. **a** Western blot analysis of p-eIF2α and ATF4. **b** Salubrinal driven elevation of osteocalcin mRNA level. **c** ATF4 level after transfecting siRNA specific to ATF4. **d** Relative mRNA levels of ATF4 in response to RNA interference with ATF4 siRNA and non-specific control (NC) siRNA. **e** Relative mRNA levels of osteocalcin (OCN). The *asterisk* is for the comparison to the control with NC siRNA, and the *dagger* is the comparison between the samples transfected with ATF4 siRNA

Inhibition of osteoclastogenesis of RAW264.7 cells by salubrinal

The primary aim of this study is to evaluate the effects of salubrinal on osteoclastogenesis. In response to 0.1–20 μM salubrinal for 24 h, we examined cell mortality and live cell numbers of RAW264.7 pre-osteoclasts. Cell mortality ratio did not present statistically significant differences in the presence and absence of RANKL (Fig. 3a). The number of live cells was increased by ~50 % by incubation with RANKL, and administration of 10–20 μM salubrinal reduced the numbers approximately by 10 % (Fig. 3b). Consistent with the stimulatory role of RANKL, the number of TRAP-positive multi-nucleated cells was substantially increased by the addition of RANKL. However, administration of 0.5–20 μM salubrinal reduced the number of TRAP-positive cells in a dose dependent manner (Fig. 3c, d).

Downregulation of NFATc1 in RAW264.7 cells by salubrinal

The NFATc1 is a transcription factor critical for activating osteoclastogenesis. Addition of RANKL to the culture medium significantly induced NFATc1 expression at day 2 and maintained its elevated level on day 4 (Fig. 4). The RANKL-induced expression of NFATc1 was reduced by administration of 5–20 μM salubrinal on both days, and the effect of salubrinal was dose dependent (Fig. 4).

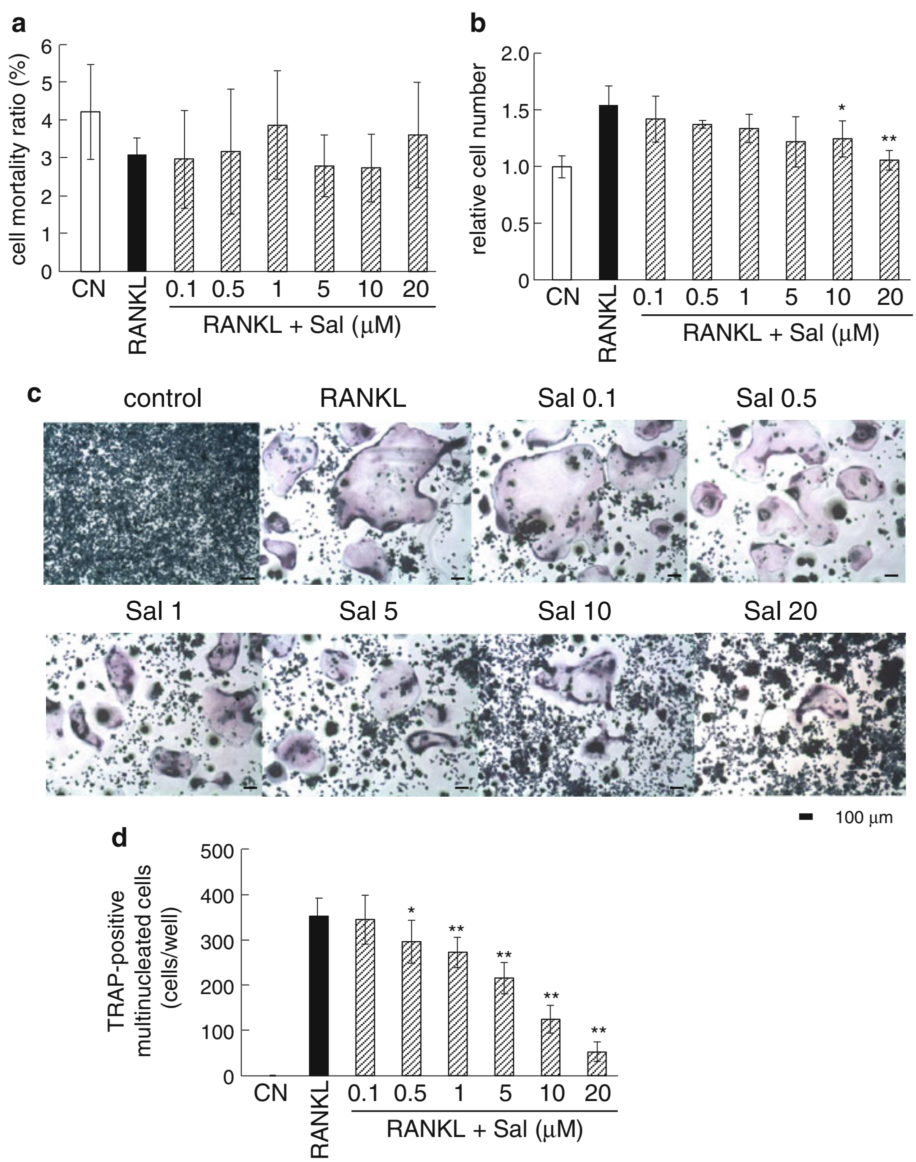

Fig. 3 Inhibitory effects of salubrinal on RAW264.7 pre-osteoclasts. *CN* control, and *Sal* salubrinal. The *single and double asterisks* indicate $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by salubrinal. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by salubrinal

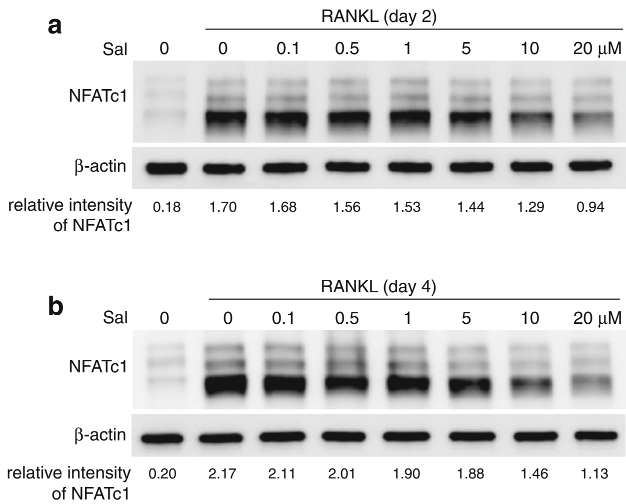

Fig. 4 Reduction of RANKL-induced NFATc1 expression by salubrinal. *Sal* salubrinal. The relative intensity of NFATc1 to β-actin is shown. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Expression of NFATc1 (4 days after RANKL administration)

Partial suppression of mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR by salubrinal

Addition of RANKL increased the mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR, and administration of 20 μM salubrinal significantly reduced their mRNA levels. On day 2, for instance, the RANKL-driven increase was 9.4 ± 0.5 fold (NFATc1), 1.9 ± 0.1 fold (c-fos), 165 ± 4.2 fold (TRAP), and 467 ± 22 fold (OSCAR). The reduction by 20 μM salubrinal was 46 % (NFATc1), 32 % (c-fos), 35 % (TRAP), and 21 % (OSCAR) (Fig. 5a). Consistent with the observed dose response, administration of salubrinal at 0.1–1 μM did not contribute to significant reduction in these mRNA levels except for NFATc1 and c-fos on day 4 (Fig. 5b).

Temporal profile of p-eIF2α and NFATc1

The temporal expression profile revealed that addition of RANKL transiently reduced the phosphorylation level of eIF2α (2–8 h) and elevated NFATc1 by 13.4 ± 3.2 fold (24 h) (Fig. 6). This induction of NFATc1 was partially suppressed by salubrinal with an increase in the level of p-eIF2α. In the early period (2–4 h), administration of 20 μM salubrinal increased the level of p-eIF2α but did not alter the level of NFATc1. In the later period (8–24 h), however, the level of NFATc1 was significantly reduced by 48 % (8 h) and 44 % (24 h). Administration of 20 μM salubrinal did not significantly alter the phosphorylation level of ERK, p38 MAPK, and NFκB (Fig. 6). Note that the normalized level of "1" in Fig. 6c was defined as the level for the cells that were not treated with RANKL without administration of guanabenz.

Inhibitory effects of guanabenz on osteoclastogenesis of RAW264.7 cells

To further examine a potential involvement of p-eIF2α in regulation of osteoclastogenesis, we employed guanabenz that also acts as an inhibitor of de-phosphorylation of eIF2α. Administration of 1 and 5 μM guanabenz did not alter cell mortality and the number of live cells, although its administration at 10 and 20 μM reduced the number of live cells in 24 h (Fig. 7a, b). Consistent with salubrinal's inhibitory action, guanabenz also attenuated osteoclastogenesis of RAW264.7 cells in a dose dependent manner (Fig. 7c, d). Compared to the number of TRAP-positive multi-nucleated cells of 377 ± 39 (RANKL only), guanabenz reduced the number of differentiated osteoclasts to 364 ± 38 (1 μM), 288 ± 51 (5 μM), 189 ± 25 (10 μM), and 73 ± 16 (20 μM).

Reduction of RANKL-induced NFATc1, c-Fos, TRAP, and OSCAR by guanabenz

The induction of NFATc1 by RANKL was suppressed by guanabenz in a dose dependent manner (Fig. 8a). The mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR were also reduced by administration of 20 μM guanabenz. Lower concentrations of guanabenz, 5 and 10 μM, were effective in reducing the levels of TRAP and OSCAR mRNA (Fig. 8b). The temporal expression profile of p-eIF2α and NFATc1 in response to 20 μM guanabenz revealed that p-eIF2α was upregulated in 2 h and NFATc1 was partially suppressed in 8 h (Fig. 9). The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of guanabenz. In the absence of RANKL administration, however, either salubrinal or guanabenz did not significantly alter cell mortality and expression of NFATc1 and TRAP (Supplementary Fig. S4).

Reduction in salubrinal/guanabenz-driven suppression of NFATc1 expression by RNA interference for eIF2α

To evaluate the effects of eIF2α on the expression level of NFATc1, we employed RNA interference specific for eIF2α together with a non-specific control (NC) (Fig. 10). In response to 20 μM salubrinal, RAW264.7 cells transfected with the control siRNA demonstrated a reduction of NFATc1 by 56 %. However, the expression of NFATc1 was reduced only by 20 % in the cells transfected with eIF2α siRNA. Furthermore, 20 μM guanabenz decreased the level of NFATc1 by 43 % in the cells transfected with

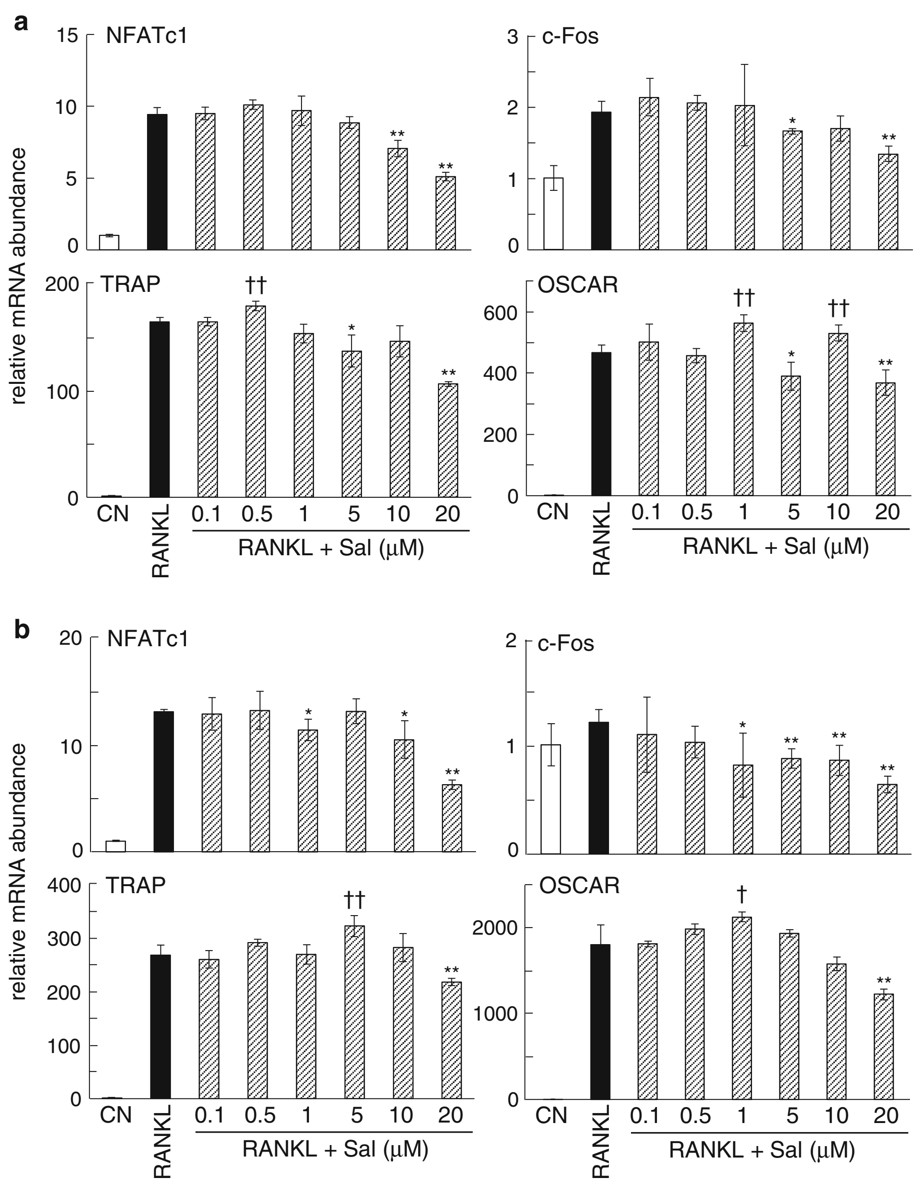

Fig. 5 Effects of salubrinal on mRNA expression levels of NFATc1, c-Fos, TRAP, and OSCAR. *CN* control. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. The *single and double daggers* indicate significant increases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Messenger RNA levels (2 days after RANKL administration). **b** Messenger RNA levels (4 days after RANKL administration)

the control siRNA but the transfection of eIF2α siRNA abolished the suppressive effect of guanabenz. The phosphorylation level of NFκB was not significantly altered by transfection with eIF2α siRNA.

Discussion

In this study we demonstrate that differentiation of RAW264.7 pre-osteoclasts to multi-nucleated osteoclasts is inhibited by administration of salubrinal and guanabenz, which block de-phosphorylation of eIF2α and elevate the level of p-eIF2α. The growth area covered by multi-nucleated cells is significantly reduced by salubrinal and guanabenz in a dose dependent manner. Partially silencing eIF2α using RNA interference significantly suppressed salubrinal/guanabenz-driven reduction of NFATc1 expression. Together with the stimulated development of MC3T3 E1 osteoblasts by an increase in ATF4 expression, the results herein suggest that eIF2α mediated signaling may play a physiological role in osteoclastogenesis and osteoblastogenesis.

Both salubrinal and guanabenz interact with PP1 and inhibit its activity of de-phosphorylating p-eIF2α. Guanabenz is reported to bind to PP1R15A subunit [14], while the exact binding site of salubrinal is not known. Guanabenz is also known as an α_2-adrenergic receptor agonist and used to treat hypertension [24]. The observed

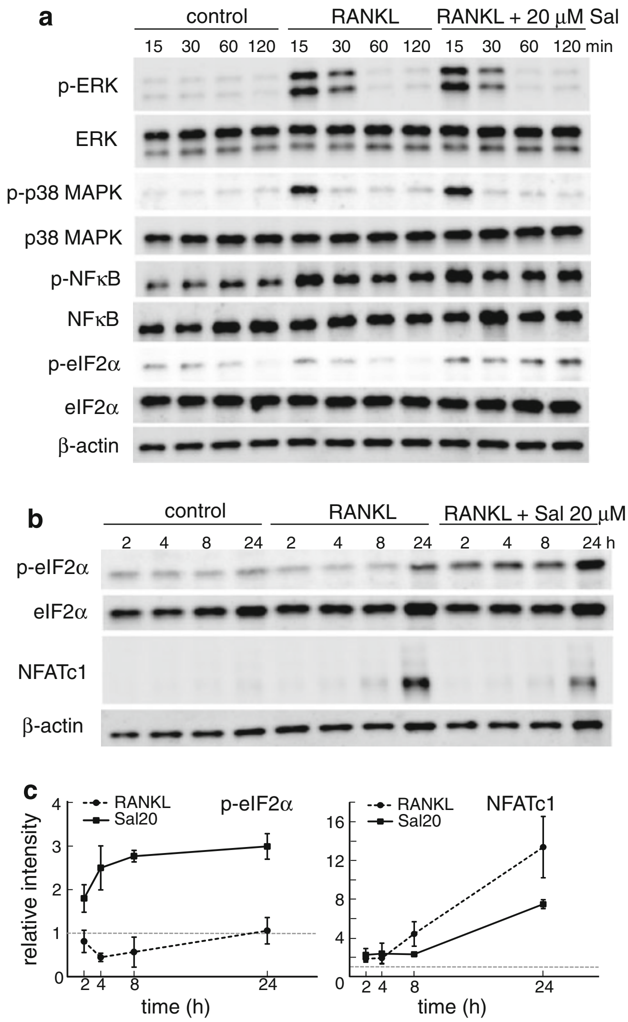

Fig. 6 Temporal expression profile of p-ERK, p-p38 MAPK, p-NFκB, p-eIF2α and NFATc1 in the presence and absence of 20 μM salubrinal. **a** Western blot analysis of p-ERK, p-p38 MAPK, p-NFκB, and p-eIF2α at 15, 30, 60 and 120 min. **b** Western blot analysis of eIF2α-p and NFATc1. **c** Comparison of the expression level of eIF2α-p and NFATc1 with and without 20 μM salubrinal. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of salubrinal

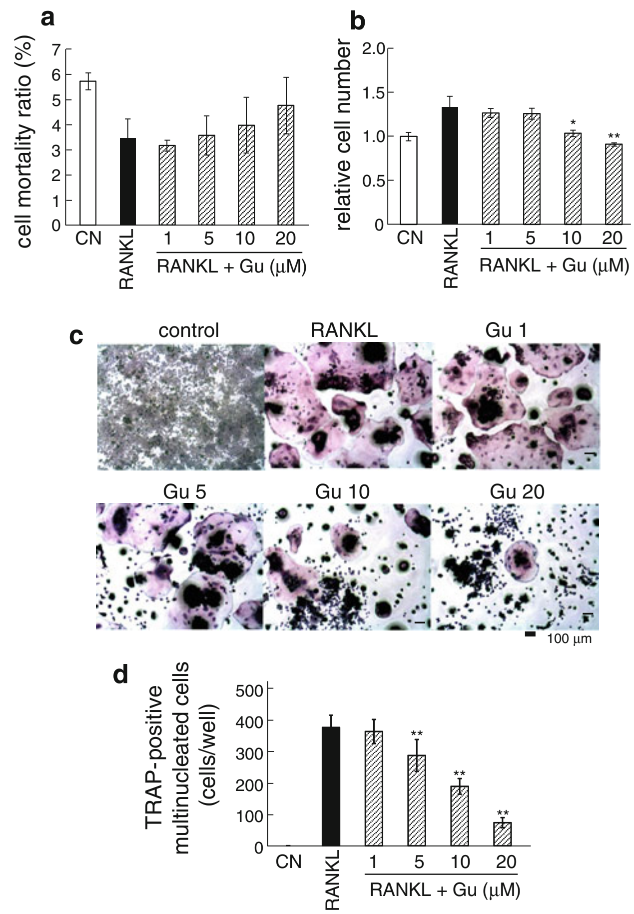

Fig. 7 Inhibitory effects of guanabenz on development of RAW264.7 pre-osteoclasts. *CN* control, *Gu* guanabenz. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by guanabenz. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by guanabenz

stimulation of osteoblastogenesis as well as attenuation of osteoclastogenesis by both agents strongly indicates that eIF2α-mediated signaling is central to regulation of ATF4 and NFATc1. This result is also supported by the salubrinal-driven alterations in the mRNA levels of osteocalcin and TRAP, which are representative in development of osteoblasts and osteoclasts, respectively. Osteocalcin is synthesized solely by osteoblasts for matrix mineralization and calcium homeostasis [25], while TRAP is highly expressed in osteoclasts and its overexpression has been observed to cause bone loss in transgenic mice [26].

The elevation of p-eIF2α is reported to enhance the development of osteoblasts and mineralization of extracellular matrix. In response to various stresses such as oxidation, radiation, and stress to the endoplasmic reticulum, cells undergo either survival or an apoptotic pathway [27]. As part of a pro-survival program, the level of p-eIF2α is raised followed by diminished translational efficiency to all proteins except for a limited group including ATF4 [5]. Salubrinal's action mimics the induction of a pro-survival program without imposing damaging stresses, which result in the upregulation of ATF4 without inducing apoptosis. Since ATF4 is a transcription factor critical for osteoblastogenesis and bone formation, we examined the effects of the administration of

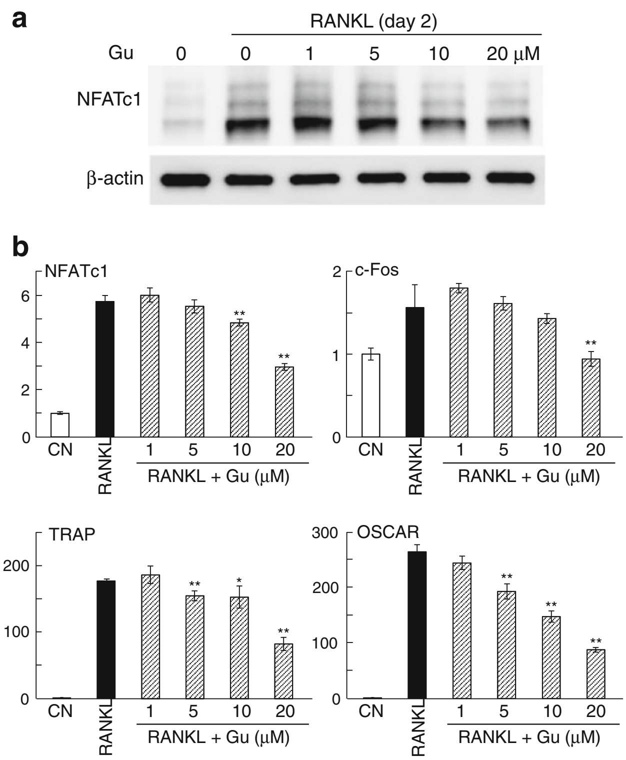

Fig. 8 Reduction of RANKL-induced NFATc1 expression by guanabenz. *CN* control, *Gu* guanabenz. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Messenger RNA levels of NFATc1, c-Fos, TRAP, and OSCAR (2 days after RANKL administration)

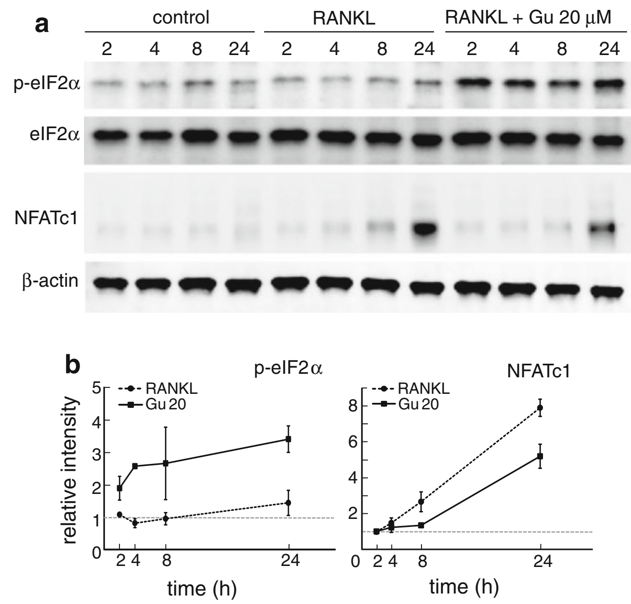

Fig. 9 Temporal expression profile of p-eIF2α and NFATc1 in the presence and absence of 20 μM guanabenz. *Gu* guanabenz. **a** Western blot analysis of eIF2α-p and NFATc1. **b** Comparison of the expression level of eIF2α-p and NFATc1 with and without 20 μM guanabenz. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of guanabenz

salubrinal and guanabenz on the mRNA level of osteocalcin as well as the mineralization of the extracellular matrix. Silencing ATF4 using RNA interference significantly suppressed salubrinal-driven upregulation of osteocalcin expression. Thus, the result here is consistent with the previously reported role of salubrinal that stimulates new bone formation in the healing of bone wound [8].

A schematic diagram illustrating the proposed pathway of eIF2α-mediated signaling in osteoblastogenesis and osteoclastogenesis is presented (Fig. 11). Through inhibition of de-phosphorylation of eIF2α, salubrinal and guanabenz are capable of enhancing bone formation by activating ATF4, as well as reducing bone resorption by down-regulating NFATc1. Osteoclastogenesis is a complex developmental process, in which active interactions take place between osteoblasts and osteoclasts. In the RANK/RANKL/OPG signaling pathway, for instance, osteoblasts provide RANKL that stimulates osteoclastogenesis. Since binding of RANKL to RANK is known to activate MAPKs and NFκB [28, 29], we evaluated a potential role of ERK, p38, and NFκB in the eIF2α-mediated signaling. In response to administration of 20 μM salubrinal, we examined the levels of p-ERK, p-p38 MAPK, and p-NFκB together with p-eIF2α. However, no detectable changes in the levels of their phosphorylated form were observed. It is possible that salubrinal may activate transcription factors such as MafB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), IRF8 (interferon regulatory factor 8), and Bcl6 (B cell lymphoma 6), which are known to be inhibitors of NFATc1 [30–32]. Alternatively, microRNA and epigenetic changes such as histone modification regulate expression of NFATc1 might be involved [33, 34]. For instance, H3K27 demethylase is reported to demethylate the site of H3K27me3 of NFATc1 and stimulate RANKL-induced osteoclastogenesis [34]. The results herein require further analysis on a regulatory mechanism that links elevation of p-eIF2α to the suppression of NFATc1.

A recent study independently reported that salubrinal alters the fate of osteoclasts and bone resorption through eIF2α-mediated translational regulation [35]. Herein, we further examined the regulatory mechanism using not only salubrinal but also guanabenz, which are the inhibitors of PP1. The results revealed that these agents can also regulate expression of NFATc1 at a transcriptional level. A separate in vivo study as well as in vitro studies using

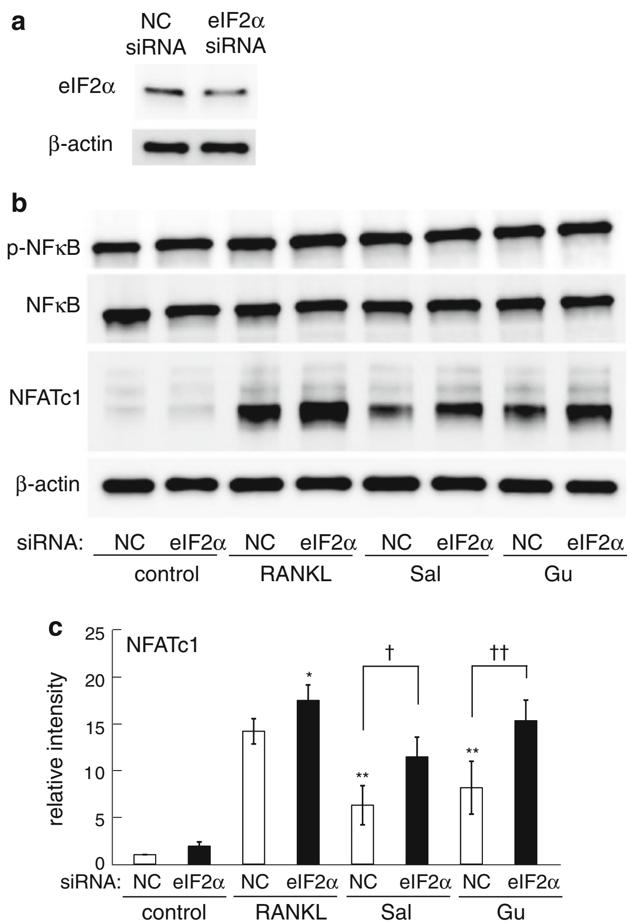

Fig. 10 Reduction in salubrinal/guanabenz driven suppression of NFATc1 expression by RNA interference specific for eIF2α. *Sal* salubrinal, *Gu* guanabenz, and *NC* non-specific control siRNA. The *single and double asterisks* indicate significant changes to the RANKL-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. The *single and double daggers* indicate significant changes to the salubrinal or guanabenz-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. **a** eIF2α level after transfecting siRNA specific to eIF2α. **b** Western blot analysis of p-NFκB and NFATc1. **c** Comparison of the expression level of NFATc1 between control siRNA and eIF2α siRNA

primary bone marrow derived cells support salubrinal's efficacy on inhibition of bone resorption. In summary, we demonstrate that elevation of p-eIF2α stimulates osteocalcin expression through upregulation of ATF4 in osteoblasts and inhibits TRAP expression via downregulation of NFATc1 in pre-osteoclasts. Silencing eIF2α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. The results in this study support the possibility of regulating bone remodeling through eIF2α-mediated signaling for combatting bone loss in osteoporosis.

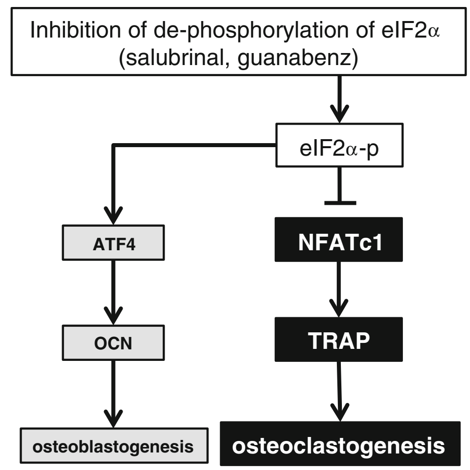

Fig. 11 Proposed mechanism of eIF2α signaling on osteoclastogenesis through NFATc1

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Conflict of interest All authors state that they have no conflicts of interest.

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Salubrinal Regulates Bone Remodeling and Fat Metabolism in Ovariectomized Mice

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Introduction: Bone remodeling is conducted by a coordinated action of osteoclasts (bone resorption) and osteoblasts (bone formation), and osteoporosis reflects imbalanced activities of osteoclasts and osteoblasts. It is recently reported that salubrinal, a synthetic chemical agent can accelerate healing of bone wounds [1]. In this study, we address a question: does salubrinal alter differentiation of bone marrow derived cells - progenitor cells to osteoclasts, osteoblasts, and adipocytes? Using ovariectomized (OVX) mice as a model for postmenopausal osteoporosis, we examined a hypothesis that salubrinal not only elevates bone mass but also reduces fat mass and restore OVX-induced obesity through modulation of the fate of bone marrow derived cells.

Methods: Thirty-six C57BL/6 female mice (~ 12 wks) were used. Twenty-four mice were subjected to OVX (OVX control group, and OVX salubrinal group at 1 mg/kg), and twelve mice were used as a sham OVX (age-match control) group. Changes in BMD/BMC in the lumbar spine and abdominal fat were measured by pDEXA. Bone marrow mononuclear cells were harvested, and we examined osteoclast (OCL) lineage (formation, migration, and adhesion) as well as colony formation of osteoclast progenitors with a colony forming unit-macrophage assay (CFU-M). Differentiation of osteoblasts was examined with a colony-forming unit-osteoblast assay (CFU-OBL).

Results: Attenuation of OVX symptoms: OVX mice presented bone loss (decrease of BMD/BMC) and obesity (data not shown). Administration of salubrinal suppressed an increase in body weight and a decrease in uterine weight (Fig. 1A & 1B).
Suppression of osteoclast differentiation: Compared to the sham OVX control, OVX activated osteoclast formation (Fig. 2A). Administration of salubrinal induced a dosage-dependent inhibition of osteoclast formation at both early and later times (Fig. 2B). Furthermore, salubrinal significantly reduced the migration and adhesion of osteoclasts (Fig. 2C&D; Fig. 3A).
Promotion of osteoblast differentiation: Besides its effects on osteoclasts, salubrinal significantly promoted differentiation of osteoblasts (Fig. 3B).
Reduction in fat and increase in BMC: Salubrinal decreased abdominal fat (%) (Fig. 3C), and increased BMC in the lumbar spine (Fig. 3D).

Discussion: The current study using OVX mice demonstrates that salubrinal regulates bone remodeling through the promotion of osteoblast differentiation as well as the reduction of osteoclast formation, migration and adhesion. Furthermore, it suppresses OVX-induced increase in body weight and fat storage. A separate study using in vitro systems indicate that besides altering the phosphorylation level of eukaryotic translation initiation factor 2a, salubrinal affects the phosphorylation of p38 MAPK and NFκB. Further studies are needed to clarify the molecular

mechanism underlying the observed effects.

Significance: Salubrinal is effective in elevating BMD/BMC and reducing fats in OVX mice, which presented osteoporosis and obesity. It could be considered as a potential therapeutic chemical agent for the regulation of bone remodeling and fat metabolism of patients with postmenopausal osteoporosis.

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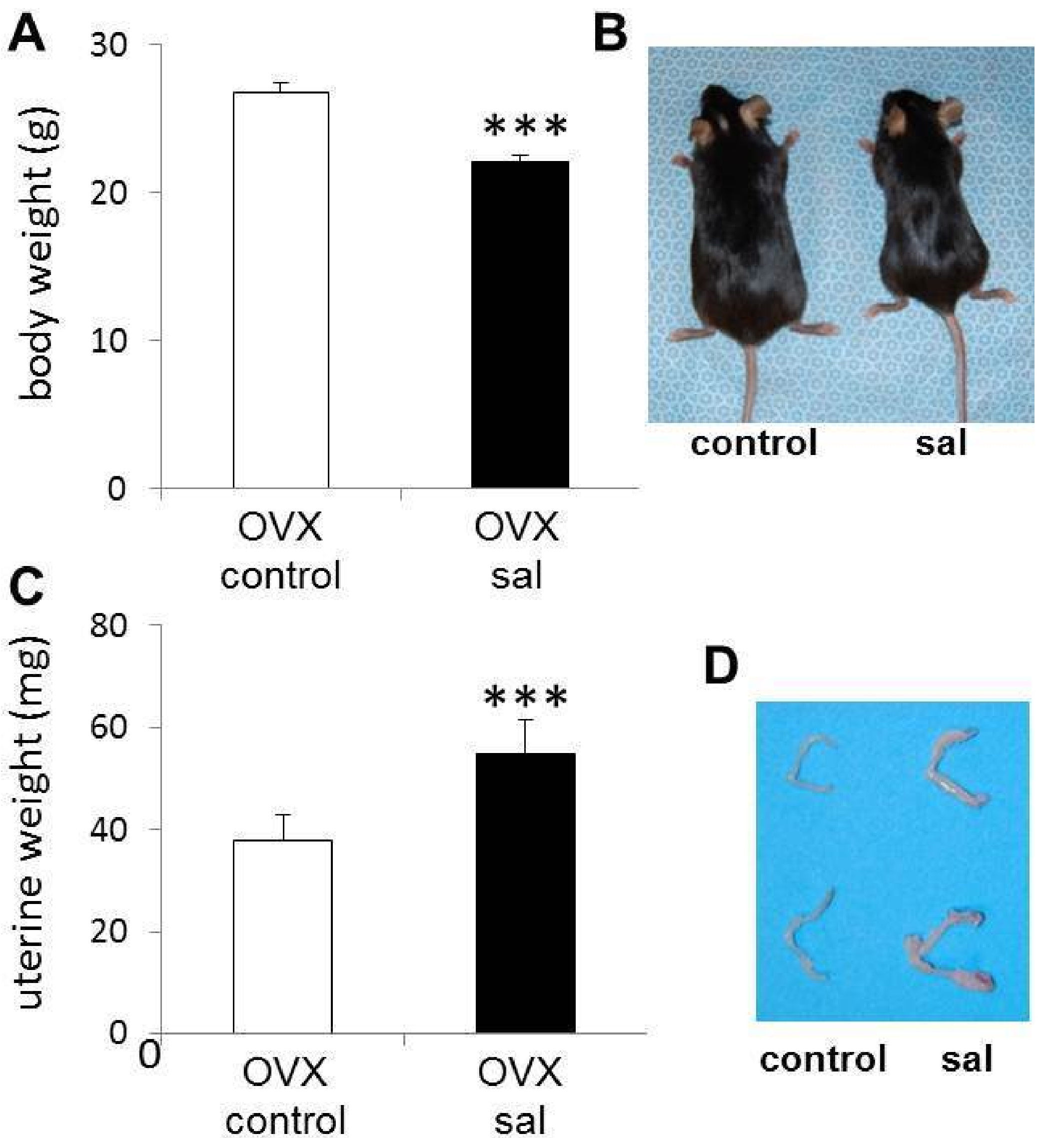

Fig. 1. Effects of salubrinal in body weight and uterine weight in OVX mice. (A-B) Reduction in body weight. (C-D) Increase in uterine weight.

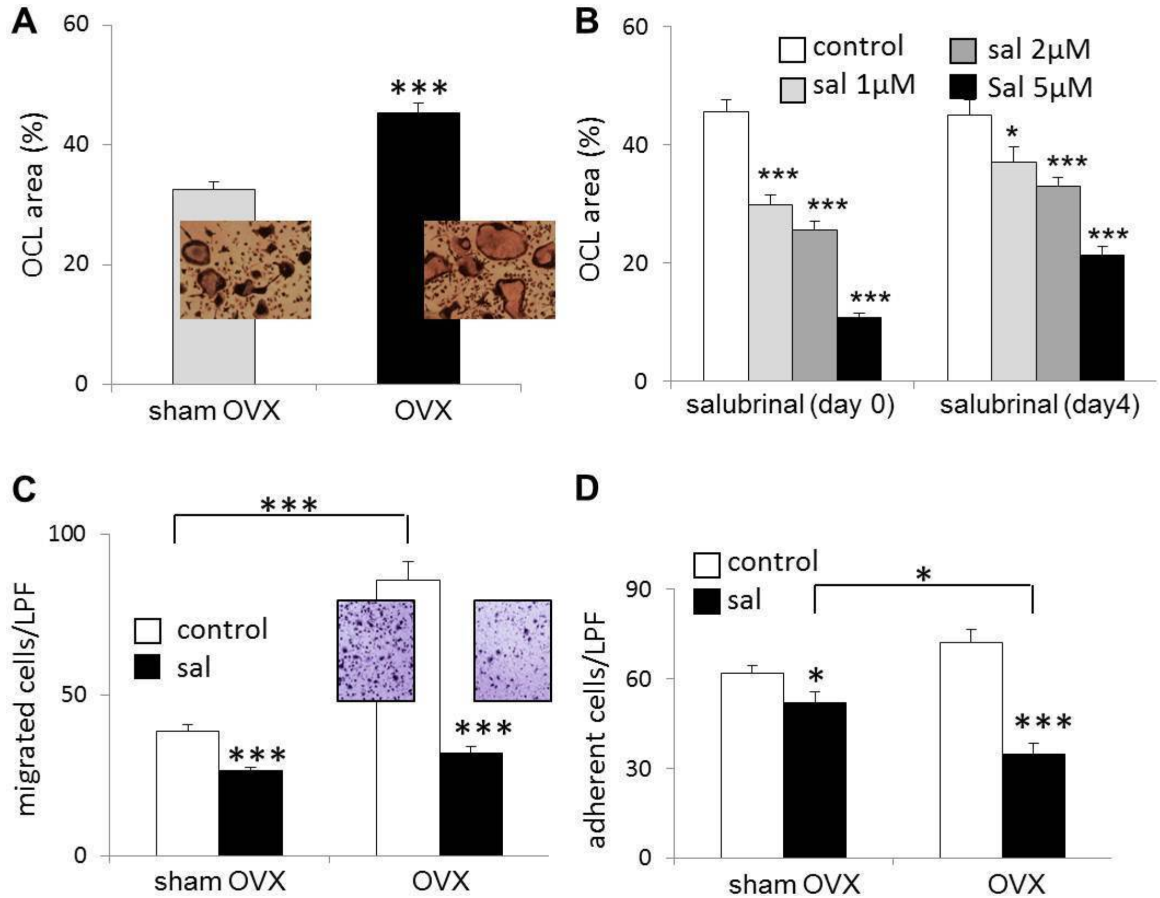

Fig. 2. Effects of salubrinal on osteoclasts (OCL) in OVX mice. (A) Increased formation. (B) Dosage-dependent reduction by salubrinal. (C) Reduction in OCL migration. (D) Reduction in OCL adhesion.

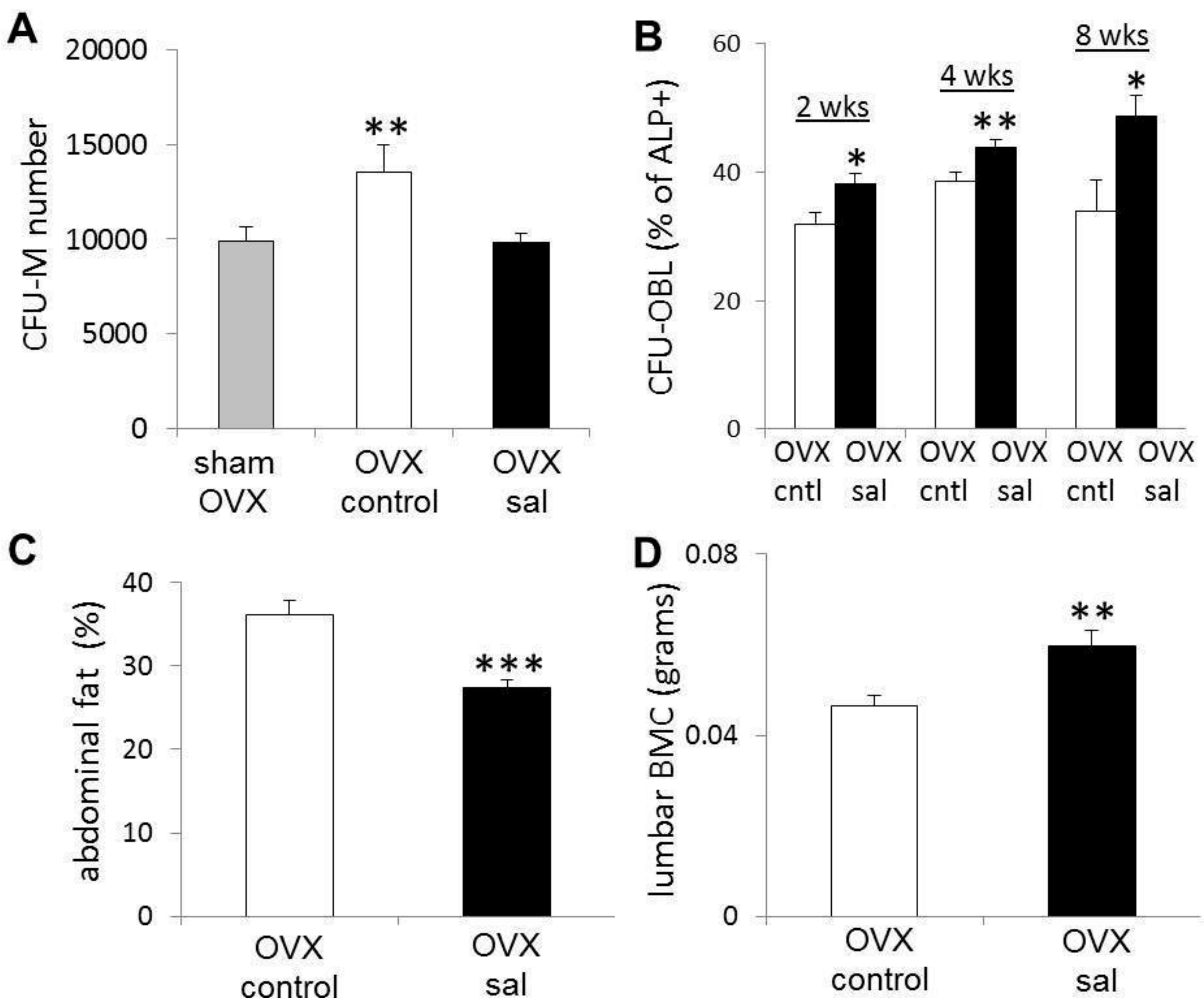

Fig. 3. Effects of salubrinal in OVX mice. (A) Reduction in CFU-M. (B) Increase in CFU-OBL. (C) Reduction in abdominal fat. (D) Increase in BMC in the lumbar spine.